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TITLE: Vaccination of High-Risk Breast Cancer Patients with Carbohydrate Mimicking Peptides

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14. ABSTRACT The expression of the Tumor Associated Carbohydrate Antigens such as the neolactoseries antigen Lewis Y (LeY) and gangliosides such as GM2 and GD2/GD3 are amplified on breast cancer cells and is linked to poor prognosis and high risk of disease relapse. Immunotherapy to direct responses to TACA is, therefore, perceived to be of clinical benefit. To overcome this deficiency, we developed mimotopes of TACA to induce more robust cross-reactive and tumor-specific responses. In preclinical studies, immunization with these mimotopes reduce tumor burden and inhibited metastatic outgrowth of murine tumor cells expressing TACA structural homologues. Thus, peptide mimotopes of TACA represent a new and very promising tool to induce a strong immune response to TACA expressed on Breast Cancer cells. Based on encouraging preclinical results, our objectives are for the current funding period (years 1 and 2) are to develop the necessary preclinical data required by the Food and Drug Administration (FDA) for filing an Investigational New Drug (IND). In this context we: 1.) Developed the necessary procedures for the required Good Laboratory Practice (GLP) studies; 2.) Defined problems in scale up of the manufactured mimotope vaccines; 3.) Identified alternative mimotopes of TACA that circumvent the scale up problems.

15. SUBJECT TERMS
1.) Developed the necessary procedures for the required Good Laboratory Practice (GLP) studies; 2.) Defined problems in scale up of the manufactured mimotope vaccines; 3.) Identified alternative mimotopes of TACA that circumvent the scale up problems.

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Introduction

Carbohydrates are the most abundantly expressed self-antigens on tumor cells and consequently they are perceived as viable targets for immunotherapy. Examples of tumor-associated carbohydrate antigens include the gangliosides GM2, GD2, GD3, and fucosyl GM1, Globo H, polysialic acid, STn and the neolactoseries antigens sialyl-Lewis x (sLex), sialyl-Lewis a (sLea) and Lewis Y (LeY). A major approach to induce responses to these tumor associated carbohydrate antigens (TACA) is carbohydrate-conjugate vaccines. Representative examples of these vaccines in clinical development include those directed toward gangliosides, polysialic acid, Globo, Lewis Y (LeY), and the STn antigen. Because TACA are T-cell–independent antigens and self-antigens, conjugation to immunologic carrier protein is perceived essential to recruit T cell help for antibody generation. Conjugation of TACA does not, however, ensure an increase in immunogenicity because conjugation strategies do not uniformly enhance carbohydrate immunogenicity. Furthermore, even with conjugation, the lack of induction of cellular immune responses that would amplify TACA-reactive humoral responses necessitates constant boosting with vaccine. Such deficiencies indicate that better ways to synthesize carbohydrate immunogens reflective of natively expressed carbohydrate structures or alternative ways to induce cross-reactive immune responses with native carbohydrate are needed.

Peptide surrogates of TACA are T-cell–dependent antigens and therefore immunization with these surrogates is predicted to facilitate cellular responses. Our rational is based upon our preclinical studies that these surrogate antigens, referred to as mimotopes, induce immune responses that inhibit tumor growth in animal models. We observe that our mimotopes 106 (GGIY<u>WRY</u>DIYWRYDIYWRYD) and 107 (GGIY<u>YRY</u>DIYYRYDIYYRYD) mimic several types of carbohydrates on breast cancer cells, principle among them is the LeY antigen. Our purpose is to induce TACA reactive antibodies in breast cancer patients by using a peptide mimotopes. We expect after vaccination to observe a robust anti-TACA response in individuals that should positively impact on tumor recurrence.

In year one AIM 1, our focus was to conduct and report preclinical studies relevant for IND filing. We had a three prong approach required for our IND filing: Develop preclinical plan, obtain FDA buy-in, and initiate preclinical studies, Develop the infrastructure required to conduct the preclinical studies and Assess any adverse immunopathology associated with the peptide mimotopes and begin scale up of the mimotope conjugated vaccine.

Body

The major goal of this application are to determine the safety and tolerability of immunization with a peptide mimotope vaccine; and to determine whether immunization with the vaccine generates a humoral response against TACAs and TACA expressing breast cancer cell lines.

We have defined three specific aims for a period of five years.

Aim 1 Conduct and report preclinical studies relevant for IND filing (Year 1-2)

A. Develop preclinical plan, obtain FDA buy-in, and initiate preclinical studies

B. Manufacture preclinical supplies of vaccine, fill/finish, stability monitoring and QA

C. Complete animal safety/toxicity studies

D. Prepare technical reports for in-house preclinical studies

E. Prepare and submit pre-IND and IND documents to the FDA

Aim2 Assure safety and determine Sufficient Immunogenic Dose (SID) of a mimotope vaccine in a Phase IA dose-escalation trial (year 2-3)

- Complete GMP manufacture, and obtain FDA and IRB approval prior to initiation Phase 1A
- Enroll and treat 18 patients (maximum) for Phase 1A safety dose-finding study
- Complete safety monitoring of mimotope vaccine
- Determine if SID has been met or repeat dose-finding for OID
- Prepare clinical study report for Phase 1A

Aim 3 Conduct a Phase IB trial to evaluate the immunogenicity of the mimotope vaccine in breast cancer patients (Year 4-5)

- Enroll and treat a total of 40 patients for Phase IB trial.
- Complete immunogenic analysis of mimotope vaccine

As this progress report concerns the first year, we will emphasize our report on Aim1.

A. Develop preclinical plan, obtain FDA buy-in, and initiate preclinical studies

Upon acceptance of the award for the "Vaccination of High-Risk Breast Cancer patients with Carbohydrate Mimicking Peptide" grant there was a lot of administrative work to be completed. Prior to the approval of the grant, pre-clinical meetings were held weekly and these meetings continue on a biweekly basis. During the pre-clinical meetings, an agenda is followed pertaining to questions, concerns and the needs of the pre-clinical phase of the grant. The meetings consist of all the staff members working on the grant and the Office of Research Compliance Quality Assurance Unit (ORC QUA).

Throughout the first year of the study there were a lot of approvals to be made for the study. The animal protocol had to be submitted and approved by the Institutional Animal Care and Use Committee (ACORP) both at UAMS and at the Veterans Administration which is adjacent to UAMS. We have completed this task and we are now able to move forward with using rodents in the study. We are currently working with the Central Arkansas Veterans Healthcare System Research and Development Committee to complete the paperwork for use of their GLP compliant facilities to conduct the pre-clinical studies portion of the grant. This process has been long, but we are reassured that the outcome of approval will be in the near future. There are small corrections to be made to the submission upon the approval of the pre-clinical protocol.

The pre-clinical portion of the grant is held under Code of Federal Regulation Title 21 Part 58 (21CFR58) Good Laboratory Practice (GLP) for Non-clinical Laboratory Studies. In an effort to make sure every aspect of the pre-clinical research portion of the grant is in compliance with 21CFR58, we had to implement standard operating procedures (SOPs) and certify that all staff members working on the study are trained.

All SOPs are written in compliance within the Code of Federal Regulation for GLP and Non-Clinical Research. There are a total of seventy-seven SOPs in place. The seventy-seven SOPs that were written include SOPs in the area of administration, animal care, equipment, histology, immunology, quality assurance and safety. The following is a list of the SOPs:

Administration
AM001 - Creation, Revision, and Implementation of SOPs
AM002 - Personnel Records
AM003 - Indexing, Handling, Storage, and Retrieval of Records and Data

EQU015 - Glassware Washer
EQU016 - Microbiologic Monitoring
EQU017 - Autoclave Gettinge Castle
EQU018 - Autoclave AMSCO
EQU019 - Microcentrifuge
EQU020 - Microm Autostainer maintenance

AM004 - Study Personnel Training
AM005 - Protocol and SOP Deviation
AM006 - Error in GLP Source Data
AM007 - Data Recording
AM008 - Specimen Shipping
AM009 - Management Responsibilities
AM010 - Archiving Original Paper Documents Electronically
AM011 - Electronic Records & Signatures

Animal Care
ANCA001 – Specimen Handling and Tracking
ANCA002 – Animal Care and Husbandry
ANCA003 – Animal Receiving
ANCA004 – Preparing an Animal room for Procedure
ANCA005 – Preparing an Animal room for receiving animals
ANCA006 – CO2 Euthanasia
ANCA007 – Rodent Quarantine
ANCA008 – Animal Feeding & Bedding
ANCA009 – Rodent Ear Marking
ANCA010 – Handling dead or moribund animal
ANCA011 – Rodent Weighing
ANCA012 – Rodent Daily room log
ANCA013 – Necropsy
ANCA014 – Cardiac
ANCA015 – Preparing Serum

Equipment
EQU001 – Balance Calibration PB602-2
EQU002 – Balance Maintenance
EQU003 – Balance Operation PB602-2
EQU004 – Autoclave/sterilization
EQU005 – Cage Wash Operations
EQU006 – Cage washroom
EQU007 – Metabolic cages
EQU008 – Disinfection of a reagent
EQU009 – Water Deliver
EQU010 – Microscope Maintenance
EQU011 – Maintenance refrigerator
EQU012 – Maintenance Tissue TEK VIP 3000
EQU013 - Wet Tissue Storage
EQU014 -Thermometer Operation Calibration
EQU021 - Boekel Scientific Lab Oven
EQU022 - Lipshaw Model 374
EQU023 - Thelco GCA Precision Scientific Oven
EQU024 - Maintenance Embedder
EQU025 - Vacuum sealer
EQU026 - Balance calibration Accu-413
EQU027 - Operation of the Accu-413 Balance
EQU028 - Balance Accu-413 Maintenance

Histology
HIST001 – H&E Autostaining
HIST002 – Coverslipping
HIST003 – Embedding
HIST004 – Tissue Collection
HIST005 – Microtome
HIST006 – Tissue processing
HIST007 – Urinalysis
HIST008 – Cryostat
HIST009 – Trimming
HIST010 – QA Histology

Immunology
IMM001 – Immunization
IMM002 – Vaccine Mixture
IMM003 – Test and Control Articles
IMM004 – FACS Protocol
IMM005 – FACS maintenance
IMM006 – Hemacytometer

Quality Assurance
QAU001 - Quality Assurance Responsibilities
QAU002 - QAU Personnel Training
QAU003 - Maintaining the Master Schedule
QAU004 - Filing and Indexing QAU Study Records
QAU005 - QAU Non-clinical Inspections

Safety
SAF001 – Handling Syringes, Needles & Sharps
SAF002 – Reporting-Tracking Work Relate Injury-illness

Once a SOP is written it goes through a process of approval. The approval process was implemented so that all SOPs are in compliance within the Code of Federal Regulation. SOPs are written by staff on the study. Staff members can make recommendations for SOP revisions at any time. Once the SOP is written, it is then submitted to the Project Program Manager (PPM) for review. Once the SOP is reviewed by the PPM it is then submitted to the Office of Research Compliance Quality Assurance Unit (ORC QUA) for review of compliance and revisions, if any. Upon completion of review by ORC QUA, the PPM will then prepare a draft of the revised procedure for review by the Author, if there are revisions to be made. Once revisions are made the SOP is sent back to ORC QUA for review. If there are no revisions to be made to the SOP it is then sent to the Study Director (SD) for approval. If approved, the SOP will be signed and dated by the Author and SD then it will be forwarded to Management (The Vice-Chancellor for Academic Affairs and Research Administration at the University) for review, approval, signature and date. If the SOP is approved by Management it is then implemented within each lab relevant to the GLP study and to the GLP study as a whole. If the SOP is not approved, it is sent back to the PPM and the approval process is repeated until the SOP is approved

The assurance of all staff members being properly trained in compliance with 21CFR 58 is critical to the GLP study (Table 1). There are rules and regulations that have to be followed under 21CFR58 and under the research administration by-laws of the University of Arkansas for Medical Sciences (UAMS), pertaining to training of research staff on a GLP study. It is mandatory for all research staff on the study to have Standard HIPPA Compliance, Human Subject Protection and HIPPA for Research for Human Subject training. Good Laboratory Practice training is also mandatory for all staff working within the GLP Lab.

Mandatory Training

Study Personnel on the Grant	**Job Title**	**Standard HIPAA Compliance training**	**Human Subject Protection Training**	**HIPAA Research for Human Subjects**	**Good Laboratory Practices**	**CV**	**Position Description**
Artaud, Cecile	Study Coordinator	03/24/06	10/27/06	10/27/06	03/03/06	On File	On File
Hennings DVM, Leah	Protocol Director	03/24/06	04/10/06	04/12/06	03/03/06	On File	On File
Hutchins MD, Laura	Clinical Director	03/24/06	02/12/06	03/03/03	N/A	On File	On File
Jousheghany, Fariba	Research Assistant	03/24/06	10/31/06	06/12/06	03/03/06	On File	On File
Karbassi PhD, Behjatolah	Immune Monitoring Director	03/24/06	10/20/06	04/24/03	03/06/06	On File	On File
Kieber-Emmons PhD, Thomas	Study Director	03/24/06	03/03/06	01/01/06	03/03/06	On File	On File
Milton, Lateefah	Project Manager	03/24/06	04/10/06	03/28/06	01/22/07	On File	On File
Siegel, Eric	Biostatistician	03/24/06	01/26/06	04/01/03	N/A	On File	On File

Breast Cancer Reseach

SOP CHECK OFF FORM

EMPLOYEE INFORMATION			
Employee Name:			
Job Title:		Manager:	

SOP DETAILS

Administration

- ☐ AM001 - Creation, Revision, and Implementation of SOPs
- ☐ AM002 - Personnel Records
- ☐ AM003 - Indexing, Handling, Storage, and Retrieval of Records and Data
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- ☐ EQU010 – Microscope Maintenance
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- ☐ EQU012 – Maintenance Tissue TEK VIP 3000
- ☐ EQU013 - Wet Tissue Storage
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- ☐ EQU016 - Microbiologic Monitoring
- ☐ EQU017 - Autoclave Gettinge Castle
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- ☐ EQU019 - Microcentrifuge
- ☐ EQU020 - Microm Autostainer maintenance
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- ☐ EQU026 - Balance calibration accu-413
- ☐ EQU027 - Operation of the ACCC-413 Balance
- ☐ EQU028 - Balance Accu-413 Maintenance

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- ☐ QAU005 - QAU Nonclinical Inspections

Safety

- ☐ SAF001 – Handling Syringes, Needles & Sharps
- ☐ SAF002 – Reporting-Tracking Work Related Injury-illness

I ___________________________ certify that I have read and understand the checked marked SOPs. I understand that I am responsible for implementing SOPs that are related to my job functions.

Staff Signature:		Date:	

In an effort to ensure all staff members are trained properly, a comprehensive file is maintained on each staff member containing documentation of all mandatory and job specific training completed and/or training that has to be completed. The staff file contains certificates of completion of training, licenses and a resume or curriculum vitae. The staff training file also includes all SOP training the staff member has completed on a SOP Employee Check-off form.

To ensure reproducibility and accuracy of our technicians work technique we performed several necropsy sessions. During these sessions two technicians practiced cardiac puncture, necropsy and gross pathology under the supervision of our veterinary pathologist. According to the veterinary pathologist, both technicians are competent and able to perform each technique with dexterity.

These sessions also allow us to ensure that all the personnel at the VA animal facility involved in our study are properly trained and that the facility possesses all the amenities required for a GLP study. We also ensure that all supplies necessary for the study are available and ready to use. Materials such as balances and flow cytometer were checked and calibrated ensuring quality assurance.

Ensuring that all relevant documentation pertaining to the study is secure was also a goal we set out to archive for the study. To ensure that all storage for paper documentation, logs and specimens are safe, we ordered special fire and water proof cabinets. All cabinets are locked and are located within a secure room. We also implemented the GLP compliant software SOPTrak designed at Baylor University. The SOPTrak software was designed to use in GLP pre-clinical studies. We use the software for archiving official electronic back-up copies of paper documents that described program activity regarding the conduct of the GLP study. The SOPTrak software and all documents within SOPTrak are stored on a secure server here at UAMS.

B. Manufacture preclinical supplies of vaccine, fill/finish, stability monitoring and QA

We previously demonstrated that mimotopes 106 (GGIY<u>WRY</u>DIYWRYDIYWRYD) and 107 (GGIY<u>YRY</u>DIYYRYDIYYRYD) mimic complex carbohydrates like LeY and simple mono -and disaccharide, components of a variety of TACA that are expressed on Breast Cancer cells. This multiple antigen mimicry (MAM) provides power to these mimotopes in that they preclude the development of multivalent vaccines encompassing multiple TACA. The mimotopes in one single immunization can induce responses to multiple TACA. Mimotope 107 in particular reacts with the lectin *Griffonia simplicifolia* lectin 1 (GS-I) and Wheat germ Agglutin (WGA) while mimotope 106 only reacts with WGA. These lectins see terminal monosaccharides. To demonstrate that autoimmunity is not an inevitable consequence of amplification of carbohydrate reactive antibodies, the immune pathology of BALB/c mice immunized with unconjugated mimotopes 106 and 107 as described in our grant, which are potent enough to induce anti-tumor response and reactive with ubiquitously expressed self carbohydrates on murine tissues was analyzed (manuscript in preparation).

Tissues from unimmunized mice were labeled with GS-I and antibody to murine IgG to demonstrate the presence of natural, circulating antibodies against terminal galactose. Western blots of membranes from murine mammary 4T1 cells, syngeneic with BALB/c mice, were compared using GS-I lectin, immunized serum antibodies, and naive serum antibodies. Tissues from immunized mice were analyzed histologically after 4 immunizations and after 1 year of immunization using hematoxylin and eosin stain, TUNEL stain for apoptosis, and Luxol-fast blue staining for myelination. ELISA against ssDNA, dsDNA, and histones was performed on sera from these mice. The pattern of expression of terminal galactose moieties is restricted and is closely paralleled by the immunoglobulin deposition pattern in unimmunized mice. There was no evidence of pathological autoimmunity in any immunized mice. Titers of clinically relevant antinuclear antibodies were not significantly elevated. These results demonstrate that vaccination with the mimotopes 106 and 107 can enhance antibodies to TACAs without inducing immunopathology. The results of these studies were presented as a Poster at the recent American Association for Cancer Research meeting in Los Angeles and a manuscript is being prepared. These studies were used to validate our SOPs as a "dry run" and will be repeated with the conjugated vaccines.

NeoMPS was contracted with to synthesize under Good Manufacture Practice (GMP), the peptide 106 and the peptide 107 with a cysteine residue at its N-terminus to allow conjugation to KLH as mentioned in our grant proposal. However, NeoMPS encountered some issues regarding the synthesis of the mimotopes 106 and 107 due to their hydrophobic property. The synthesis procedure took some time and effort before succeeding to a final product of a lower purity than expected (91.7% v.s >95%) as indicated on the Certificate of Analysis (see appendices). Biosyn Corp. was then asked to couple the mimotopes to KLH. However, the conjugation turned out to be difficult in the scale up due to the difficult property of the peptides. We are still working on these peptides to improve their solubility for eventual coupling to KLH using a series of adapter molecules that link to the cysteine. The adaptor molecules would then be used to couple to KLH.

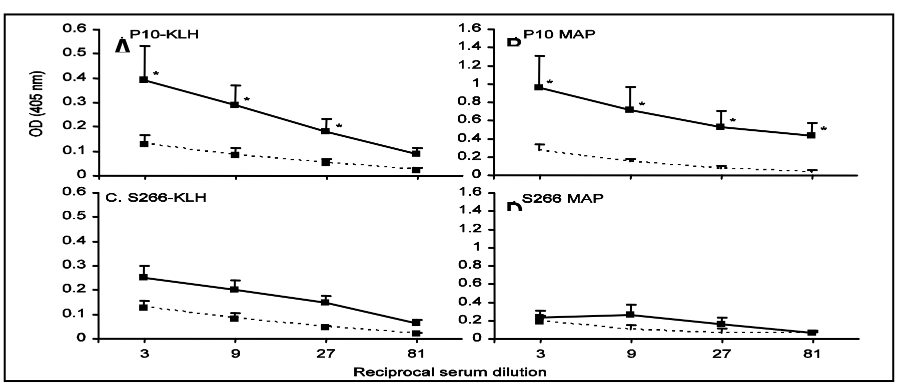
As an alternative approach we tried to improve the immunogenicity/solubility of our LeY mimotopes by linking them to a PADRE (PAn-DR Epitope) sequence instead of coupling them to KLH as mentioned in our grant proposal. It has been suggested that linear constructs, containing the PADRE epitopes, might be effective at generating an immune response as large multivalent antigens. This property represents a significant feature of PADRE, suggesting its potential utility as a carrier to induce T cell "help" in vaccine constructs designed for human use. Along with the increase of immunogenicity of our construct, this new strategy would allow us to decrease the cost of the synthesis procedure. However, due to the hydrophobic property of the PADRE-mimotope construct its synthesis turned out to be very difficult and almost impossible to perform.

These observations prompted us to conduct, some new experiments on related peptides as we discussed in the proposal. We looked in our peptide library and decided to evaluate the candidature peptide P10 (GVV<u>WRY</u>TAPVHLGDG) as a MAM, targeting gangliosides and LeY on breast cancer cell lines.

P10 peptide (GVV<u>WRY</u>TAPVHLGDG) possesses in its sequence the WRY motif, also present in the peptide 106 (GGIY<u>WRY</u>DIY<u>WRY</u>DIY<u>WRY</u>D). We observed that P10 binds to the anti-LeY mAb, BR55-2 and competes with LeY for BR55-2 binding just like the 106 and 107 peptides. This observation lead us to evaluate its candidacy as a LeY mimotope. We were also attracted by the fact that P10 peptide has a better solubility property than 106 and that it has been previously conjugated to KLH, ruling out our doubt on the feasibility of the conjugation procedure.

P10 was first isolated against an anti-GD2 ME361 mAb using a peptide phage display library. We showed that the binding reactivity of pre- and post-immune sera from mice immunized with P10KLH (Fig. 1 A), MAP (Fig. 1 B) showed substantial increase (>3x) in serum antibody binding to GD2 after immunizations as compared to before immunization at most serum dilutions tested. Mice immunized with S266 control peptide did not have GD2-binding serum antibodies (Fig. 1 C,D).

Fig 1. GD2-specific antibody responses after Immunization of mice with KLH-coupled peptides or MAP



P10-KLH (Fig. 2A), similar to P10-MAP (Fig. 2B), significantly ($p<0.05$ and 0.01) inhibited tumor growth. Moreover, P10-KLH, of all 4 peptides tested, was the most effective in inhibiting tumor growth in the prophylactic setting. Furthermore, P10-KLH was capable of inhibiting the growth of established D142.34 tumors.

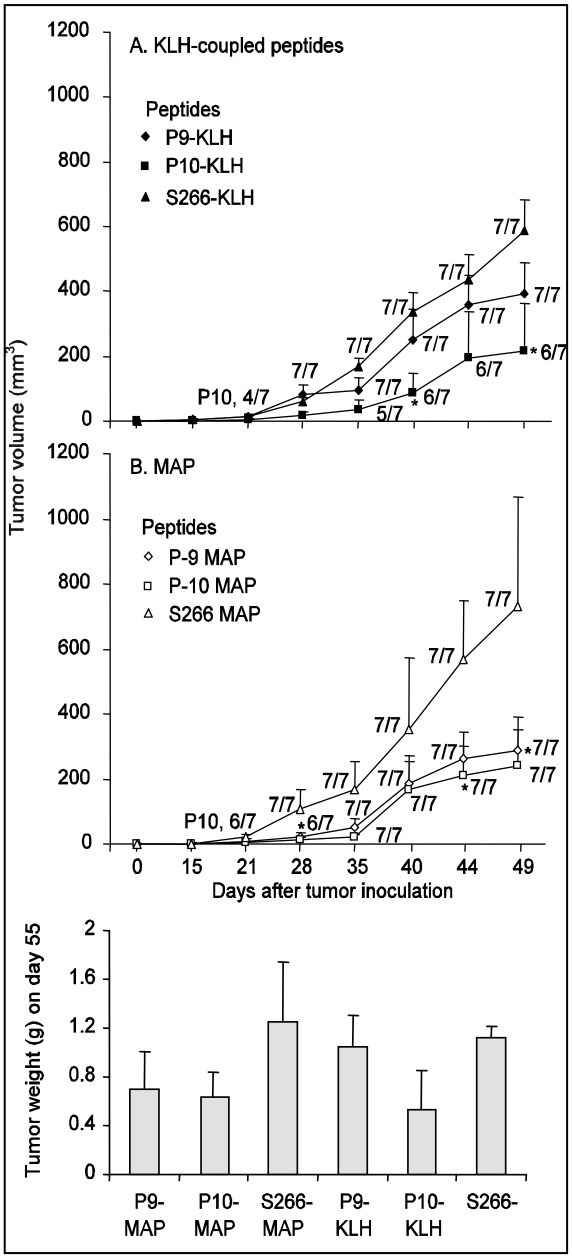


Fig 2. Inhibition of tumor growth in peptide-immunized mice
KLH-coupled and MAP peptides were evaluated for their capacity to inhibit D142.34 ($GD2^+$) melanoma growth in mice (Fig. 2).
Tumor volumes were measured on various days after tumor inoculation (Fig. 2A and B). In addition, mice were sacrificed on day 55, and tumors were excised and weighed. Tumor volumes were significantly ($p<0.05$ and <0.01) inhibited between days 35 and 49 of measurements by immunization with P10-KLH as compared to immunization with control peptide (Fig. 2A).

Fig 3 shows that there was a marginally significant (p=0.077) difference between the percentage of tumor-free mice in the P10-KLH-immunized versus S266-KLH-immunized group.

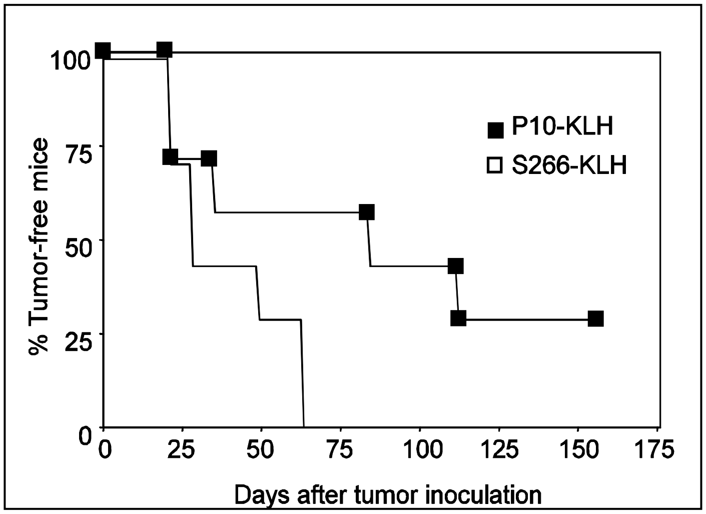


Fig 3. Inhibition of tumor growth in P10 peptide-immunized mice
C57BL/6 mice (7/group) were inoculated s.c. with 1.5×10^5 D142.34 ($GD2^+$) tumor cells. Ten days later, mice were immunized s.c. 4 times at 2-week intervals with 50 µg of GD2-mimicking peptide P10 or with control peptide S266, coupled to KLH using QS-21 adjuvant.

Similar results were observed with mice immunized with P10S-PADRE. The PADRE (PAn-DR Epitope) sequence was chosen as an alternative to KLH as described above.

Analysis of the structutre of the Mab ME361 suggest that this antibody might bind to both GD2 and GD3 antigens and that peptides that are reactive with this antibody through a defined set of hydrogen bonds might elecit antibodies with broader ganglioside reactivities. To determine if increasing the level of mimicry by P10 can enhance the anti-ganglioside respone to we perfomed structural modeling analysis of P10 binding to ME361 which suggested that the residues GVV, RYTA, VH and D were bound by ME361. Yet we observed that only two hydrogen bonds were in common with how ME361.1 binds to GD2 (Table 2). By shortening P10 peptide we have developed a peptide with a sequence WRYTAPVHLGD (referred to as P10 short or P10s). This peptide shows an increase number of hydrogen bonds relative to its parent peptide GVVWRYTAPVHLGDG (referred to as P10). The redesigned P10s shares 5 hydrogen bonds with GD2 in binding to ME36.1. Docking calculations indicate that the topographical binding mode of the P10s peptide overlaps that of GD2 in the ME36.1 combining site (Fig 4).

Table 2 Modification of flanking residues enhances GD2 mimicry for ME36.1 binding

Ligands [1]	ΔG (free binding energy)	RESIDUES ON ME36.1						
GD2	-10.06 kJ/mol	H/Thr33	H/His35	H/Asp50	H/Asn52	H/Asn59	L/Tyr93	H/Ser100
GVVWRYTAPVHLGDG	-17.60 kJ/mol	H/Thr33						H/Ser100
WRYTAPVHLGDG	-50.00 kJ/mol	H/Thr33		H/Asp50		H/Asn59	L/Tyr93	H/Ser100

[1] *The CMP residues forming hydrogen bonds with ME36.1/GD2 contact residues are underlined.*

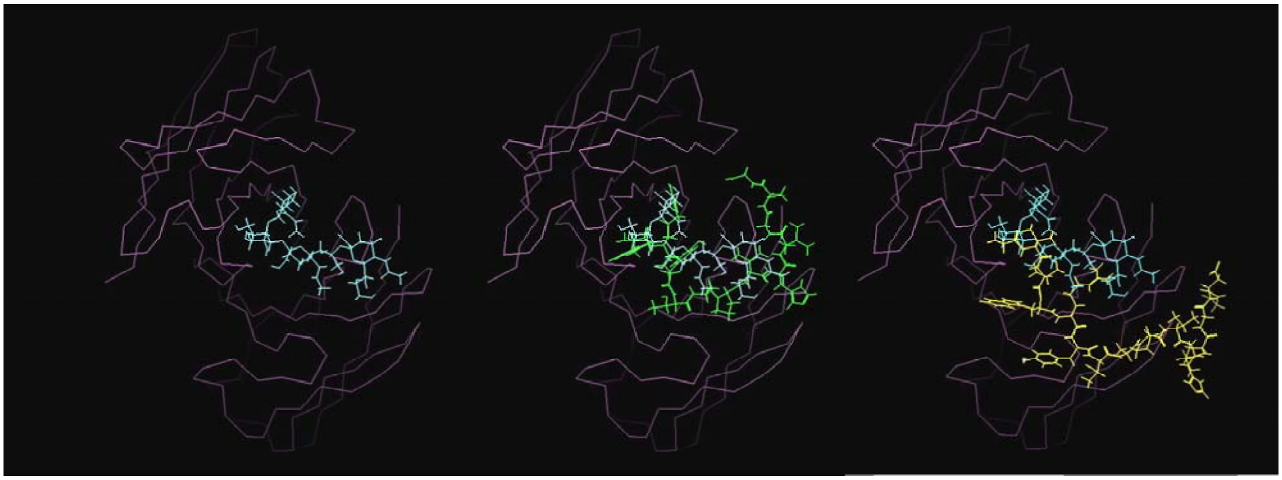


Figure 4. P10s overlaps with GD2 in the ME36.1 combining site. *Projected ME36.1 binding to GD2 in blue (left panel) relative to P10s peptide in green (central panel) and with P10 in yellow (right panel). CMP positions result from extensive*

We observed that immunization with MAP-P10S induced **serum IgM antibodies** superior in GD2 binding than serum antibodies induced by P10 (Fig. 5). Thus, the increase in the level of GD2 mimicry was translated into improved immunogenicity.

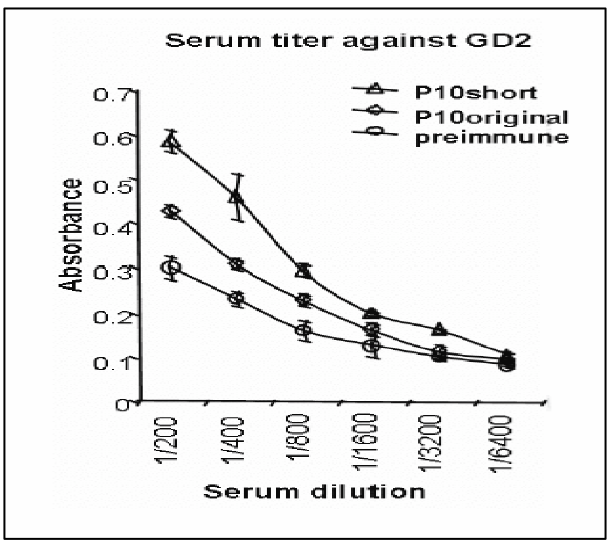


Figure 5. Immunization with P10s results in improved GD2 reactivity. *Immunization with improved peptide (P10short) induced IgM antibodies that bound to GD2 with endpoint titer 1:3200 compared to 1:800 for the original P10 peptide. The sequence improvement led to a 4-fold increase in anti-GD2 titer. Endpoint titer was determined as the serum dilution with absorbance values at least 2 SD higher than preimmune serum.*

Furthermore, P10s induced antibodies highly reactive with the GM2 ganglisodie (Fig 6) that is overexpressed on the cell surfaces of a number of human cancers, including breast cancer.

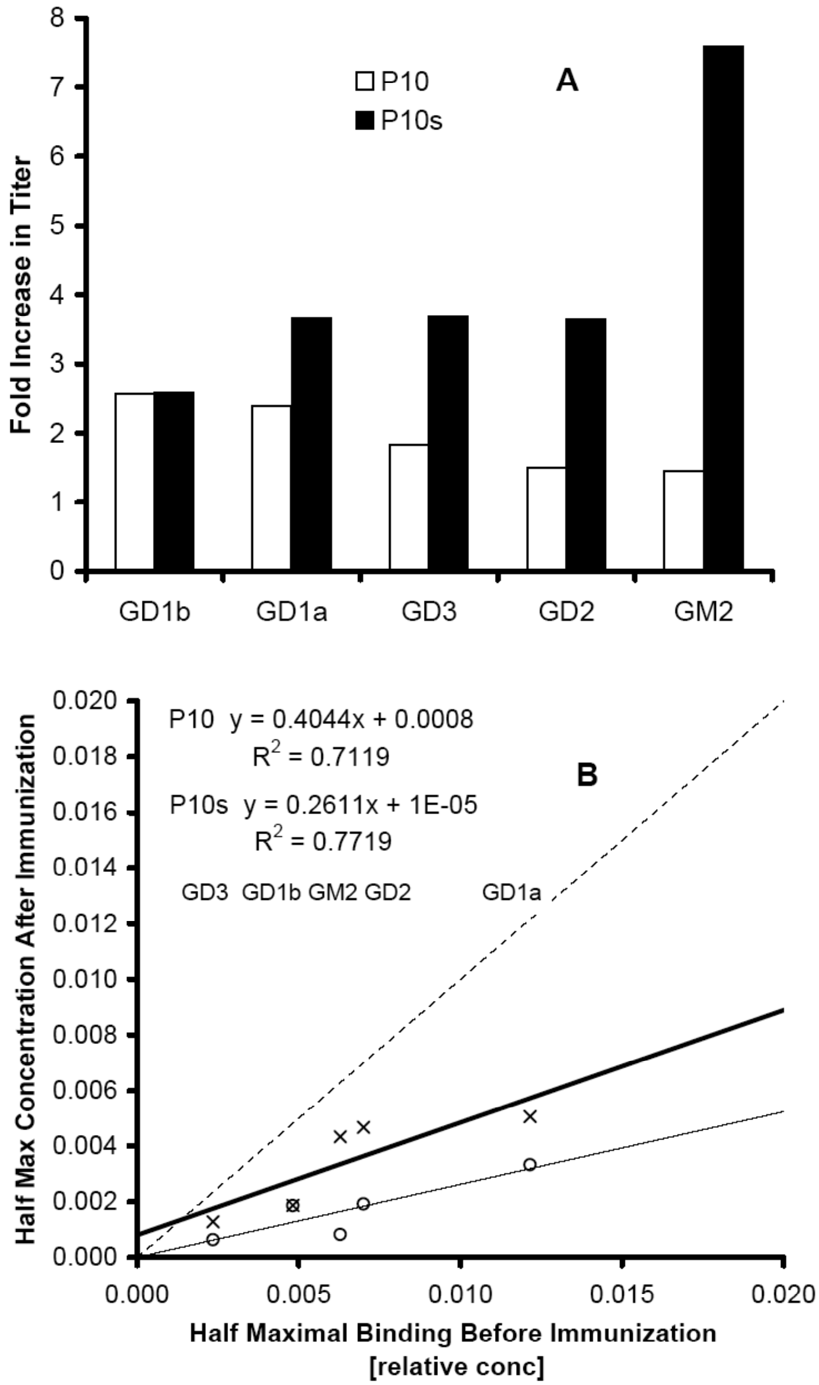


Figure 6. Binding of serum IgM antibodies to gangliosides from mice immunized with P10 or P10s as well as from naïve mice. A- comparison of dilutions yielding half-maximal binding to different gangliosides. B – correlation between reactivity of naïve and immune sera to different gangliosides. The diagonal line marks the positions of the points indicating that immunization would not change the titer. The lines below this diagonal indicate increase in titer due to immunization. The slope of the line may be used as a measure of the sensitivity of the immune titers to the preimmune titers. x are data points for P10 and o are data points for P10s.

Fig 7 shows that mice immunized with P10S-PADRE present an increase survival time compared to non-immunized animals.

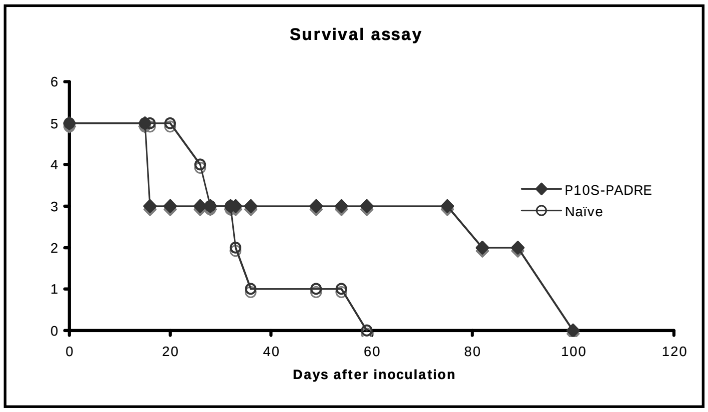


Fig 7. Inhibition of tumor growth in P10s peptide-immunized mice. C57BL/6 mice were immunized four times s.c with P10S- PADRE. 7 days after the last boost, mice were inoculated i.v. with 5x105 EL4 tumor cells.

P10 as a LeY mimotope

Herein, we are showing that P10L and P10S peptide bind with the monoclonal antibody BR55-2 (anti-LeY) (Fig 8). Confirming the observation that BR55-2 mAb binds to peptides containing a WRY motif.

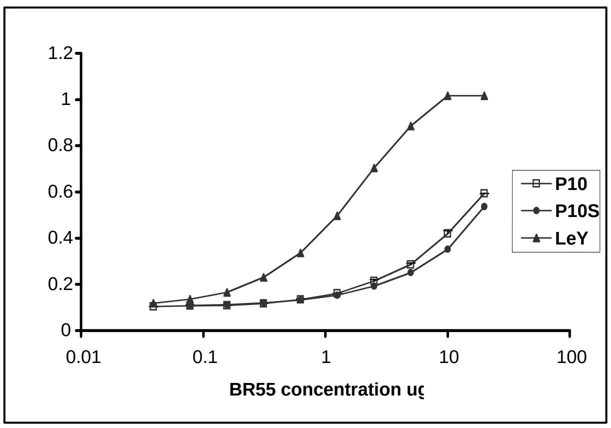
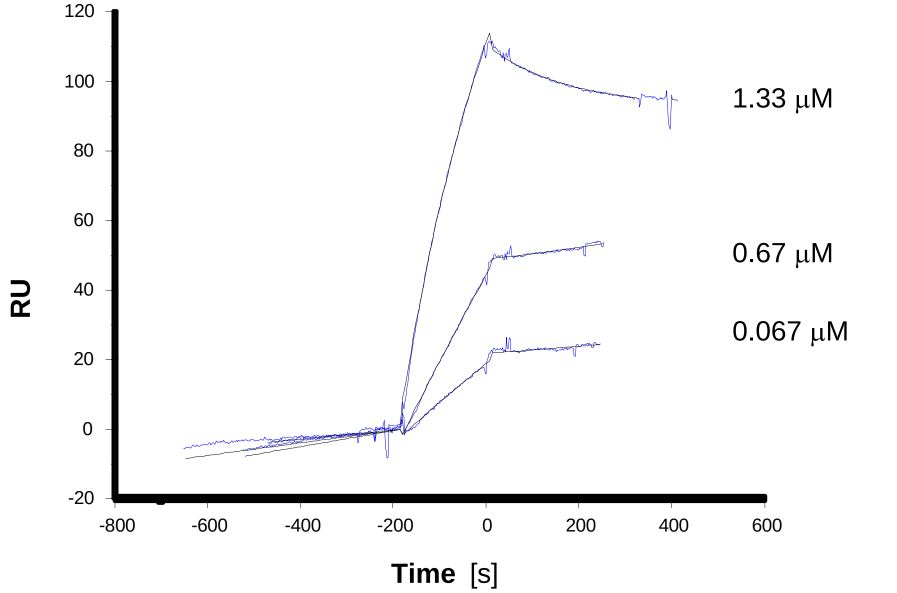


Figure 8. BR55-2 mAb binding to P10L peptide and LeY antigen. ELISA plate was coated with the peptide mimotopes P10L, P10S or LeY antigen and binding of BR55-2 was visualized by HRP-conjugated anti-mouse IgG

However in surface plasmon resonance assays P10s displays better binding than P10 (Fig 9).



k_{a1} = 885 ± 37.6 $M^{-1}s^{-1}$ k_{d1} = 1.88 ± 0.25 x 10^{-3} $M^{-1}s^{-1}$

k_{a2} = 21.9 ± 3 x 10^{-5} $RU^{-1}s^{-1}$ k_{d2} = 5.16 ± 1.1 x 10^{-3} s^{-1}

KD of a single binding site Š 2.12x10^{-6} M

Fig. 9. SPR analysis of the binding of BR55-2 to P10s. The peptide in MAP format was immobilized (350 RU) on a C1 chip to avoid interaction of the peptide with the dextran matrix. The antibody was injected over the surface at different concentrations. The sonograms were analyzed using BIAevaluation software ver. 3.1 and a bivalent analyte with drifting baseline model was applied to fit the data (chi square = 0.54). It is possible that the presentation of the MAP peptide in this setting facilitated cross

More importantly, the serum antibodies induced by P10L and P10S are capable of binding to LeY antigen directly by Enzyme-linked immunosorbent assay (ELISA) (Fig 10) and can cross-react with cancer-cells expressing LeY Antigen in a titer dependent manner (Fig11).

Figure 10. Serum binding to LeY antigen.
Serum was obtained after the third immunization with 50ug of P10 (S or L) peptide

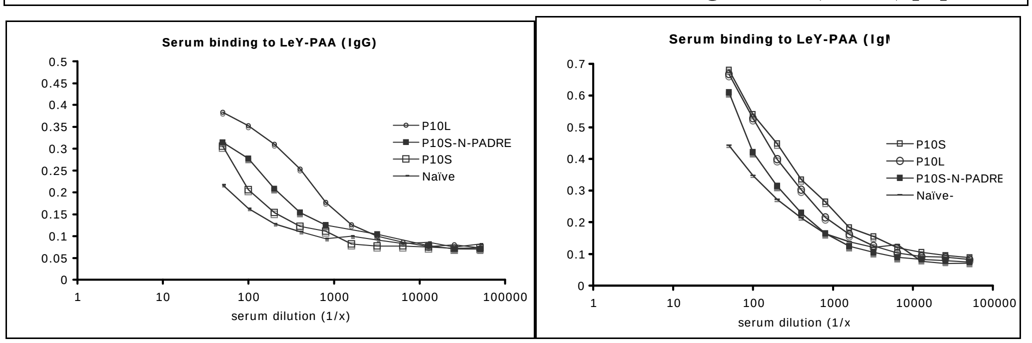
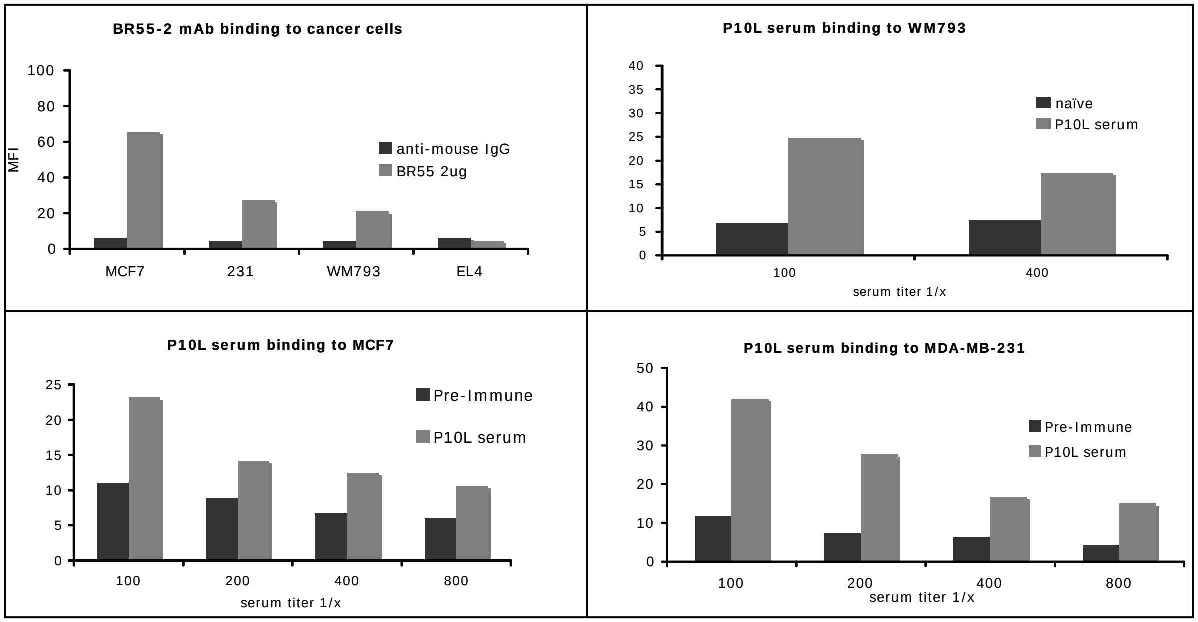


Figure 11. P10L serum binding to several different cancer cell types.

Cells were incubated with different titer of P10L serum and visualized by FITC-conjugated anti-mouse IgM or with 2ug of BR55-2 mAb and visualized by FITC conjugated anti-mouse IgG.



We also found that the serum antibodies induced by P10L and P10S are capable of mediating CDC in WM793 cells (Fig 12).

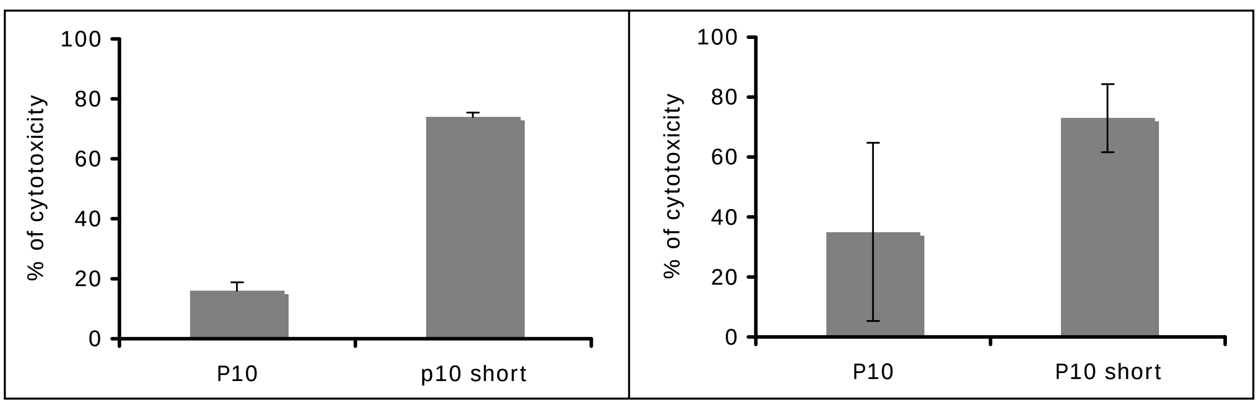


Figure 12. CDC mediated in LeY-expressing MW793 cells.
Cells were incubated in presence of P10 serum (1/50 titration) and with 1/4 (a) or 1/8 (b) rabbit complement for 4h.

According to the results presented herein, peptides P10 and P10s are viable candidates for targeting TACA on Breast Cancer cells and therefore we have decided to switch our preclinical studies to these mimotopes. We are awaiting results from ongoing immunizations with these two mimotopes (2 months) before we finalize our protocols and begin our final preclinical study for the FDA. As PADRE forms induce in vivo immune responses we are thinking of switching our formulation as well, which would simplify the manufacturing process.

C. Complete animal safety/toxicity studies and D. Prepare technical reports for in-house preclinical studies

We could not initiate the study as we could not manufacture the peptide 106 vaccine due to its difficult solubility property. We are however, optimistic concerning P10 peptide and thus relying on it to accomplish the further objectives.

E. Prepare and submit pre-IND and IND documents to the FDA

This is on going. We are developing the IND template and have written the consent form and IRB protocol. We are waiting on the final information from our immunization studies on P10 forms to finalize the pre-IND and IND documents.

KEY RESEARCH ACCOMPLISHMENTS:

<u>Manufacture preclinical supplies of vaccine</u>

1. We worked on the immunogenicity of the peptide 106. We tried to improve it by linking 106 to a Pan-DR Epitope or/and by using an alternative adjuvant (Montanide ISA51) however due to 106 difficult solubility properties we could not use any of these two strategies.
2. NeoMPS synthesized the peptide 106 and obtained 91.7% purity (see appendix).
3. Biosyn corp. could not solubilize 106 and therefore could not couple it to KLH.
4. NeoMPS synthesized the P10 peptide and obtained 97% purity in the initial run.

<u>Peptide characterization</u>

5. We have presented a 106 related peptide known as P10 (and P10s) and we showed that this peptide presents a dual mimotope function (GD2 and LeY mimotope) (see comparison table appendix)
6. We showed that the peptide P10 (and P10s) could replace the peptide 106 for the preclinical study. We established that these peptides are mimotopes of the LeY antigen. These peptides binds to BR55-2, an anti-LeY monoclonal antibody. Serum antibodies generated in mice immunized with these peptides are capable of recognizing LeY antigen and can mediate CDC on cancer cells.
7. P10 peptide has been couple to KLH in the past and is more soluble than peptide 106. Both peptides induce anti tumor responses when synthesized with PADRE as immunogenic carrier. 106 solubilities has been an issue for its synthesis and coupling to KLH. P10 and P10s peptides are easier to synthesize and to couple to KLH or synthesize with PADRE.

<u>Develop preclinical plan, obtain FDA buy-in, and initiate preclinical studies</u>

8. The Institutional Animal Care and Use Committee (ACORP) of the VA approved our Animal protocol.
9. The IND template is written and IRB protocol and consent forms have been written with the original 106 peptide but is easy to modify when our final formulation is set.

10. An archiving system has been implemented. We implemented the GLP compliant software SOPTrak. Special fire and waterproof cabinet have been ordered and installed.
11. Seventy-seven Standard Operating Procedures have been put in place in compliance within the Code of Federal Regulation Title 21 Part 58.
12. All staff members involve in the GLP study have been adequately trained.
13. A comprehensive file was created and is maintained on each staff member containing documentation of all mandatory and job specific training completed and/or training that has to be completed to ensure that all staff members involve in the GLP study have been trained properly.
14. All supplies have been ordered and ready to be use.

REPORTABLE OUTCOMES:

Results demonstrating that vaccination with the mimotopes 106 and 107 can enhance antibodies to TACAs without inducing immunopathology were presented as a Poster at the recent American Association for Cancer Research meeting in Los Angeles and a manuscript is being prepared.

Carbohydrate mimetic peptides induce tumor-associated carbohydrate-reactive antibodies in the absence of pathological autoimmunity. Leah Hennings, Cecile Artaud, Fariba Jousheghany, Marie Chow, Behjatolah Monzavi-Karbassi, Thomas Kieber-Emmons. Manuscript in preparation.

Preclinical studies of carbohydrate mimetic peptide vaccines for breast cancer and melanoma. Monzavi-Karbassi B, Hennings LJ, Artaud C, Liu T, Jousheghany F, Pashov A, Murali R, Hutchins LF, Kieber-Emmons T. Vaccine. 2007 Apr 20;25(16):3022-31.

CONCLUSION

In year one, we had a three prong approach required for our IND filing: Develop preclinical plan, obtain FDA buy-in, and initiate preclinical studies, Develop the infrastructure required to conduct the preclinical studies and Assess any adverse immunopathology associated with the peptide mimotopes and begin scale up of the mimotope conjugated vaccine.

Within this year we have developed the infrastructure required to conduct the preclinical studies. The animal protocol (ACORP) was accepted; the animal housing followed the rules and regulation of the 21CFR part 58; all Standard Operating Procedures are in place; all research staff is adequately trained. All the supply necessary for the study is available and ready to use.

However, because of the difficulties of obtaining a GMP grade 106 peptide, we could not go forward with its synthesis and coupling strategy. Thus, we have characterized the peptides P10 and P10s as substitutes for peptide 106 and peptide 107. P10 and P10s show dual properties, they are mimics of LeY and breast associated gangliosides and both display good solubility properties. Thus, we are relying on P10 and P10s to accomplish our preclinical objectives.

We have written a manuscript describing that immunization with carbohydrate mimotopes does not induce immunopathology. These results were obtained in a research setting facility with research grade peptide, and lead us to believe that no adverse immunopathology will be associated with GLP or GMP grade peptide mimotopes under the regulation of the 21CFR58 of the pre-clinical study.

Appendices

Comparison table for P106; P107 and P10

	Lectins binding to peptides		**mAb binding to peptides**	**Serum binding to breast cancer cell lines**			**Serum binding to CH**	**In vitro Functional assay**		**In vivo Experiments**		
Peptide	*WGA*	*GS1*	*BR55-2*	*231*	*4T1*	*MCF7*	*LeY*	*CDC*	*Apoptosis*	*Tumour growth inhibition*	*Increase survival rate*	*DTH*
106	+	-	+	+	+	+	+	+ (MCF7 & MethA)	-	+ (4T1)	+ (4T1)	-
107	+	+	+	+	+	+	+	+ (MCF7 & MethA)	+ (DNA format; 4T1)	+ (4T1)	+ (4T1)	-
P10	+	+	+	+	-	+	+	+/- (WM793) on going	-	+ (EL4)	+ (EL4 & tgGD2+)	+ (GD2+)

NeoMPS, Inc.
GROUPE SNPE

Certificate of Analysis (Revised)

This certifies that the peptide was tested and found to have the following results:

Lot Number: **WF213 // 196188-16 (12-17)**

Name: **UAMS**

Grade: **Research Grade**

Sequence: **H-Cys-Gly-Gly-Ile-Tyr-Tyr-Arg-Tyr-Asp-Ile-Tyr-Tyr-Arg-Tyr-Asp-Ile-Tyr-Tyr-Arg-Tyr-Asp-NH2**

Counter Ion: **Trifluoroacetate**

TEST	METHOD	SPECIFICATIONS	RESULTS
Appearance	Visual Observation	White to off-white powder	White Powder
Identity	Mass Spectrometry (MS)	MW (average) = 2855.19 MW (monoisotopic) = 2853.28	$(M+3H)^{3+}/3 = 953.1$ After deconvolution: MW = 2856.3
	Sequencing	MS/MS	Correct sequence
	Amino Acid Analysis (AAA)	Asx 3 Cys 1 Gly 2 Ile 3 Arg 3 Tyr 9 Report Results	Asx 3.2 Cys 0.2 Gly 1.9 Ile 2.7 Arg 3.1 Tyr 8.8 Note: Cys partially destoyed during hydrolysis for AA analysis
Purity Assay	RP-HPLC (Area Normalized)	> 85%	>86.30 %
Trifluorocetate Content (TFA)	Ion Chromatography-Suppressed Conductivity	Reports Results	14%
Net Peptide Content (NPC)	AAA	Report Results	80%
Water Content (H_2O)	Karl Fischer USP <921>	Report Results	1%
Total Mass Balance	Calculation NPC + TFA + H_2O	90-105%	95%

Comments:

For more information on our analytical systems, please contact our Customer Services department.

Quality Assurance by [signature]

Date 2/1/07

9395 Cabot Drive, San Diego, California 92126 Tel: (858) 408-0808 or (800) 338-4965
Fax: (858) 408-0799 or (800) 654-5592 website: www.neomps.com e-mail: mps@neomps.com

NeoMPS, Inc.
GROUPE SNPE

Certificate of Analysis (Revised)

This certifies that the peptide was tested and found to have the following results:

Lot Number: **WF212 // 196189-01**

Name: **UAMS**

Grade: **Research Grade**

Sequence: **H-Cys-Gly-Gly-Ile-Tyr-Trp-Arg-Tyr-Asp-Ile-Tyr-Trp-Arg-Tyr-Asp-Ile-Tyr-Trp-Arg-Tyr-Asp-NH2**

Counter Ion: **Trifluoroacetate**

TEST	METHOD	SPECIFICATIONS	RESULTS
Appearance	Visual Observation	White to off-white powder	White Powder
Identity	Mass Spectrometry (MS)	MW (average) = 2924.3 MW (monoisotopic) = 2922.3	$(M+3H)^{3+}/3 = 976.0$ After deconvolution: MW = 2925.0
	Sequencing	MS/MS	Correct sequence
	Amino Acid Analysis (AAA)	Asx 3, Cys 1 Gly 2, Ile 3 Arg 3, Trp 3 Tyr 6 Report Results	Asx 3.2, Cys 0.1 Gly 2.0, Ile 2.7 Arg 3.0, Trp ND* Tyr 5.8 Note: Trp and Cys partially destoyed during hydrolysis for AA analysis
Purity Assay	RP-HPLC (Area Normalized)	> 85%	> 91.7 %
Trifluorocetate Content (TFA)	Ion Chromatography-Suppressed Conductivity	Reports Results	12%
Net Peptide Content (NPC)	AAA	Report Results	83%
Water Content (H_2O)	Karl Fischer USP <921>	Report Results	1%
Total Mass Balance	Calculation NPC + TFA + H_2O	90-105%	96%

Comments:

For more information on our analytical systems, please contact our Customer Services department.

Quality Assurance by [signature]

Date 2/1/07

9395 Cabot Drive, San Diego, California 92126 Tel: (858) 408-0808 or (800) 338-4965
Fax: (858) 408-0799 or (800) 654-5592 website: www.neomps.com e-mail: mps@neomps.com

NeoMPS, Inc.
GROUPE SNPE

Certificate of Analysis

This certifies that the peptide was tested and found to have the following results:

LOT #:	XS1-53 // 287041-07(8-9)	QUANTITY:	25 mg
NAME:	UAMS, Univ of Arkansas	GRADE:	95 %

SEQUENCE: H-CGVVWRYTAPVHLGDG-NH2

RP-HPLC ANALYSIS:

Peptide is >96% pure by HPLC

(See attached representative chromatogram)

MASS ANALYSIS:

Molecular Weight: 1729.0

$(M+2H)^{2+}/2$ = 865.5

MW after deconvolution: 1729.0

(See attached spectrum)

COMMENTS:

Counter Ion: Trifluoroacetate
Appearance: White Powder

For more information on our analytical systems, please contact our Customer Services department.

Quality assured by ____________ Date 4/3/07

9395 Cabot Drive, San Diego, California 92126 Tel: (858) 408-0808 or (800) 338-4965
Fax: (858) 408-0799 or (800) 654-5592 website: www.neomps.com e-mail: mps@neomps.com

University of Arkansas for Medical Sciences
4301 W. Markham Street (slot 824)
Little Rock, AR 72205

Grant BC050157: Vaccination of High-Risk Breast Cancer Patients with Carbohydrate Mimicking Peptides: PROTOCOL 5-06-2 Determination of the safety and tolerability of immunization with a LeY peptide mimotope vaccine in mice.

University of Arkansas for Medical Sciences
4301 W. Markham Street (slot 824)
Little Rock, AR 72205

TABLE OF CONTENTS

University of Arkansas for Medical Sciences
4301 W. Markham Street (slot 824)
Little Rock, AR 72205

1. Purpose

To determine the safety and tolerability of the administration of immunogenic mimetic peptides, which are designed to induce immune responses specific for tumor associated carbohydrate antigens (mimotopes), in mice.

2. Background

We have defined a series of carbohydrate mimetic peptides (mimotopes) that induce tumor-directed, carbohydrate-reactive humoral and cellular responses in experimental animals. Preclinical data demonstrate immunization with mimotopes reduces tumor burden and prolongs survival in mice. This safety and tolerability study in mice is a required step towards developing these molecules for clinical use as anti-tumor therapies for patients with breast cancer, with the ultimate goal of impacting relapse and prolonging survival.

3. Sponsor

University of Arkansas for Medical Sciences (UAMS)
4301 West Markham St. Slot 813
Little Rock, AR., 72205

4. Testing Facility and Key personnel

a. Animal Facility

Veterinary Medical Unit (VMU)
Central Arkansas Veteran's Healthcare System
4300 West Seventh Street Research 151
Little Rock AR 72205 USA

b. Histopathology Facility

UAMS - Arkansas Cancer Research Center-ACRC, Room 429
4301 West Markham St. Slot 725
Little Rock, AR., 72205

c. Key personnel

Study Director: Dr. Thomas Kieber-Emmons, PhD
Veterinary Pathologist: Dr. Leah Hennings, DVM

5. Identification and test control substances

a. Test Substance

NeoMPS Inc (San Diego, CA 92126 · USA) will synthesize the LeY mimotope with an additional Cysteine group at the NH2-terminus to allow coupling to KLH (Keyhole limpet hemocyanin). Coupling will be performed using the MBS ((m-Maleimidobenzoyl-N-hydroxysuccinimide Ester) method

Name: KLH-106 mimotope
Peptide Seq: Cys-GGIYWRYDIYWRYDIYWRYD

Name: KLH-107 mimotope
Peptide Seq: Cys-GGIYYRYDIYYRYDIYYRYD

b. Limulus Amoebocyte lysate (LAL); Endotoxin Assay

The purpose of this endotoxin assay is to ascertain whether or not the test article is contaminated with gram-negative bacterial endotoxin. A sample of KLH-106peptide and KLH-107peptide mixed to QS21 adjuvant will be sent to Clongen Laboratories, LLC (Germantown, MD 20874), which will perform the assay under GLP conditions following the protocol CB125. A standard curve will be used to derive endotoxin values in the test article(s). Clongen Laboratories, LLC will provide a final study report, including evaluation of the results, to the study director.

c. Adjuvant

QS-21 is an immunological adjuvant. Immunological adjuvants can modulate the humoral (i.e., stimulation of antibody quantity, avidity, affinity, persistence, and/or isotype switching) and/or cellular [(i.e., stimulation of delayed-type hypersensitivity and cytotoxic T lymphocytes (CTL)] immune responses to vaccine antigens. QS-21 has been shown to stimulate both humoral and cell-mediated immunity.

Corporate Office
630 Fifth Avenue Suite 2100
New York, New York 10111

QS-21 is a naturally occurring saponin molecule purified from the South American tree *Quillaja saponaria* Molina. It is a triterpene glycoside with the general structure of a quillaic acid 3, 28-O-bis glycoside with the formula $C_{92}H_{148}O_{46}$, and a molecular weight of 1990 Kd. QS-21 will be supplied in solid powder in an amber glass vial containing at least 100 mg or 1.0 gram of QS-21.

d. Substance Storage

The KLH-106-mimotope and KLH-107-mimotope will be received in liquid condition and will be stored frozen at ≤-20º C for maximum stability.

QS-21 will be stored at ≤ -20°C. The shelf life at this storage condition is four (4) years. The expiration date for solid powder QS-21 is listed on the vial label and on the certificate of analysis.

Temperature logs will be maintained and recorded on business days from date of receipt. Logs will be treated as raw data.

6. Test System

a. Test system characteristics

Test system: Mouse

- **Number of animals**: 168 mice
- **Body weight range**: 15-25 grams
- **Sex**: Female

Strain: Balb/c
Age of the test system upon receipt: 3 to 4 weeks
Source of supply:

Charles River Laboratories International, Inc.
Corporate Office
Attn: Research Models and Services
251 Ballardvale Street
Wilmington, MA 01887-1000

b. Test System Justification

The preferred animal model for toxicity testing is an animal expressing the relevant tumor antigen. The neolactoseries antigen LeY is not expressed in mice but a structurally related difucoganglioside, also mimicked by mimotope 106 and 107, is endogenously expressed on murine tumors. Therefore, we propose a preclinical safety study to provide a gross characterization of the nature, frequency, and severity of adverse responses observed following vaccine administration in this tolerant mouse setting. The preclinical study will provide an initial basis upon which to determine the vaccine safety profile in a manner to support further study, including Phase 1 clinical testing.

c. Group assignment/control of Bias

Upon receiving, each animal will be assigned randomly to a cage. The randomization schedule for assignment to peptide treatment, sacrifice time, and urine-collection caging are shown in Tables A, B, and C (see Annex A, B and C), respectively, and were generated by a block randomization scheme implemented in Microsoft® Office Excel 2003. Block sizes were 4 for both Peptides, 3 for sacrifice times, and 8 for urine-collection caging. Animals will be ear punched according to the group assignment tables (Table-A see Annex).

7. Experimental Design

a. Formulation preparation

Lab personnel will wear suitable protective clothing such as laboratory coat, and gloves according to UAMS policy.

The vaccine shall be prepared according to the SOP IMM002. Briefly, 20µg of QS-21 per mouse shall be mixed with the appropriate quantity of peptide in 200µl sterile phosphate buffered saline.

The syringe should be loaded with the vaccine mixture in the animal procedure room just before its use.

b. Immunization procedure using adjuvant / peptide mixture

i) **Animals**

Female, 3-4 weeks old Balb/c mice will be purchased from Charles River

Laboratories Inc. Upon arrival the mice will be quarantined for two weeks (Standard Operating Procedure ANCA007). The animals will be housed in the animal facility located at the Veterinary Medical Unit of the VA hospital. The animals receiving and care will be conducted according to Standard Operating Procedure ANCA003 and ANCA002 respectively.

Animals will be housed in group cages holding no more than 4 mice/cage according to Standard Operating Procedure ANCA002. The animals will be identified using an ear notching system and according to Standard Operating Procedure ANCA009. A log assigning animal notch number and cage number to their respective group will be maintained (see Annex, Table-A*)*.

For 106 mimotope:
There will be 3 test and 1 control group. 24 mice will be assigned per dosing group (3 time points with 8 mice sacrificed per time point). The study groups are as follows:

Adjuvant control: 20µg QS-21 per mouse.
KLH-106 mimotope/adjuvant (Vaccine): 100 µg, 300 µg, 500 µg

For 107 mimotope:
There will be 2 test and 1 control group. 24 mice will be assigned per dosing group (3 time points with 8 mice sacrificed per time point). The study groups are as follows:

Adjuvant control: 20µg QS-21 per mouse.
KLH-107 mimotope/adjuvant (Vaccine): 300 µg, 500 µg

The total number of animals will be 168 mice.

ii) Immunization Schedule and dosing

Note: Immunization, weight measurement and observations should be done in the animal procedure room and follow the study calendar **Table 1.**

Animals in each group will be injected subcutaneously with control or test article on weeks 1, 2, 3, 7, and 19 of the study. 20µg/mouse QS-21 in saline buffer alone will be used as the control.

	EVENT	DOSES	STUDY WEEK																		
			1	2	3	4	5	6	7	8	9	10	11	12	13-18	19	20	21	22	23	24
Peptide 106	Immunization	500 ug/ml	24	24	16				16							8					
		300 ug/ml	24	24	16				16							8					
		100 ug/ml	24	24	16				16							8					
		adjuvant	24	24	16				16							8					
	Euthanasia	500 ug/ml			8						8							8			
		300 ug/ml			8						8							8			
		100 ug/ml			8						8							8			
		adjuvant			8						8							8			
Peptide 107	Immunization	500 ug/ml				24	24	16				16							8		
		300 ug/ml				24	24	16				16							8		
		adjuvant				24	24	16				16							8		
	Euthanasia	500 ug/ml						8						8							8
		300 ug/ml						8						8							8
		adjuvant						8						8							8

Three doses of vaccine will be used; 100 µg; 300 µg; 500 µg.
All immunizations will be performed according to the Standard Operating Procedure IMM001.

After the immunizations are completed, syringes will be discarded in a biohazard disposal container. (Standard Operating Procedure SAF001).

iii) Immune response assessment

Blood will be collected by cardiac puncture according to the SOP ANCA014. Sera will be then process according to SOP ANCA015.

For each cage, about 50µl of serum from each mouse will be pooled and used to assess its reactivity against LeY antigen.
The different serum will be tested against known human and murine breast cancer cell line such as MDA-231, MCF7, MethA, 4T1 using the **FACS** (Fluorescence Activated Cell Sorting) technique.
Binding will be compared to non-immunized mice serum.

Cell line	Species	Type
MDA-MB-231	Human, Caucasian	Breast, adenocarcinoma
MCF7	Human, Caucasian	Breast, adenocarcinoma
MethA	Mouse	fibrosarcoma
4T1	Mouse	Mammary carcinoma

8. In-Life Observations

a. General health monitoring

All animals will be observed daily by experienced VMU animal care staff to assess their health and well-being. Visual inspection, as to general appearance of mice and condition of bedding, will be performed per Standard Operating Procedure ANCA002. The research assistant will specifically monitor the animals three times a week for injection site redness, swelling, heat, ulceration, or hair loss. Any abnormality will be noted in the animal examination record (see Annex).

b. Weight measurement

Upon arrival at the animal care facility from the Charles River Laboratories Inc., mice will be weighed according to Standard Operating Procedure ANCA003. Animals will be weighed on a weekly basis thereafter on a calibrated scale per Standard Operating Procedure ANCA011. Weights will be recorded on the animal experimental record. (see Annex).

c. Morbidity

Appropriate assessment techniques will include: evaluation of overall clinical condition including appearance, posture, body temperature, behavior and physiological responses; assessment of food and water intake; and weighing to determine changes in body weight. Animals that become moribund or lose greater than 10% of body weight over a 2 week period during the study will thereafter be euthanized and necropsied.

d. Clinical Pathology

Urine will be collected for three days in individual metabolic cages according to Standard Operating Procedure EQU007 and prior to scheduled necropsy for complete urinalysis. Five out of eight mice per group will be chosen randomly using a Microsoft® Office Excel 2003 Randomization spreadsheet for urinalysis testing. (see Annex, Table-C).

The following parameters will be evaluated under GLP conditions at:

Rodent Clinical Pathology Core Laboratory
Central Arkansas Veterans Healthcare System
Research Services
4300 W. 7th St.
Little Rock, AR 72205

Appearance
Volume
Specific gravity
pH
Ketones
Bilirubin
Glucose
Occult blood
Urobilinogen

Blood will be collected via cardiac puncture immediately postmortem, according to SOP ANCA014.

The following parameters will be evaluated under GLP conditions at the Rodent Clinical Pathology Core Laboratory:

Central Arkansas Veterans Healthcare System
Research Services
4300 W. 7th St.
Little Rock, AR 72205

Leukocyte count, total and differential
Erythrocyte count
Hematocrit
Hemoglobin
Mean corpuscular hemoglobin, mean corpuscular

volume, mean corpuscular hemoglobin concentration (calculated)
Platelet count

Alkaline phosphatase
Aspartate Aminotransferase
Bilirubin, total
Calcium
Chloride
Creatinine
Gamma glutamyl transferase
Glucose
Lactate Dehydrogenase
Magnesium
Phosphorus
Potassium
Sodium
Total Protein
Urea Nitrogen

e. Necropsy

On Week 3, prior to injection, and on weeks 9 and 21, two cages of 4 mice per group will be chosen according to the Microsoft® Office Excel 2003 randomization spreadsheet (see Annex, Table-B) to be euthanized. Mice will be euthanized via an overdose of CO_2 (Standard Operating Procedure ANCA006) until movement and respiration cease. Death will be determined by lack of movement, heart-beat and respiration. Animals will be necropsied according to Standard Operating Procedure ANCA013.

Necropsy should be performed upon sacrifice or unscheduled death with recording of organ weights and gross pathology and preservation of a complete list of tissues at necropsy under Standard Operating Procedure ANCA013.

Tissues evaluated for organ weight:

Kidneys (paired)	Spleen
Liver	Heart

See Annex for organ weighing form.

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Little Rock, AR 72205

Tissues evaluated for gross lesions and preserved in 10% neutral formalin:

Gross lesions	Mesenteric lymph nodes
Clitoral gland	Ovaries
Brain and pituitary gland	Pancreas
Cecum	Rectum
Colon	Salivary glands, left and right Parotid, Sublingual, Submaxillary
Duodenum	
Esophagus	Skeletal muscle, quadriceps, left
Eyes, left and right	Skin, ventral and dorsal
Femur, left	Spinal cord in vertebral column
Injection site(s)	Spleen
Heart	Stomach
Ileum	Submandibular lymph nodes, left and right
Jejunum	Thymus
Lacrimal glands, left and right	Thyroids, left and right
Adrenal glands left and right	Tongue
Kidneys left and right	Trachea
Liver	Urinary Bladder
Lungs	Uterus
Mammary gland	Vagina

f. Tissue Processing

Tissues will be collected and processed according to Standard Operating Procedure HIST004 and HIST006. All harvested organs will be embedded in paraffin blocks according to Standard Operating Procedure HIST003. Tissue from the control and high dose (500 μg) group will be sectioned according to Standard Operating Procedure HIST008 and stained with hematoxylin and eosin (H&E) according to Standard Operating Procedure HIST001. Slides will be identified according to Standard Operating Procedure ANCA001 and examined by a veterinary pathologist. All gross lesions and target tissues will be evaluated in the mid- and low-dose groups.

9. Data analysis

a. Data recording

Appropriate entries in the experimental record should be made after each procedure and according to Standard Operating Procedure AM007 (See Annex for mice experimental record.)

b. Protocol and Standard Operating Procedure Deviation

All deviations to the Protocol or Standard Operating Procedure are to be reported immediately to the Study Director. All study staff are required to fill out a Protocol or Standard Operating Procedure Deviation form (see annexD and E)if he/she deviates from the implemented Protocol or Standard Operating Procedures. All Protocol and Standard Operating Procedure Deviation forms are to be sent to the Study Director and the Project Manager according to Standard Operating Procedure AM005.

c. Evaluation of Test Results

Note: All gross lesions and all tissues from 8 mice in the highest dose group and per time point will be evaluated histologically and compared with all lesions and tissues from 8 adjuvant immunized control animals. If pathologies are noted in mice in the 500µg dose group compared to control animals, all tissues from the 300µg group will be evaluated. If pathologies are noted in this group, all tissues from the 100µg group will be evaluated. Animal weights and organ weights will be summarized within each group as medians and quartiles, and compared across groups using scatterplots in conjunction with Wilcoxon rank-sum tests. Abnormalities in blood, urine, wet tissue, and the histology slides will be summarized by location as number and percent present in each group, and compared across groups using barcharts and/or profile.

d. Reports

A draft report will be provided to the sponsor for review. The final study report, including evaluation of the results, will be signed by the Study Director and veterinary pathologist and provided to the Sponsor.

e. Records and Archives

All raw data, records, protocol and report copies will be maintained according to standard operating procedure Standard Operating Procedure AM003.

f. Regulatory requirement and good laboratory practices

This study will be conducted in compliance with the U.S FDA Good Laboratory Practice Regulations (21CFR58) and according to standard operating procedures.

9. Confidentiality

All information regarding the identity of the test substance and data are considered to be confidential. No raw data, worksheets, data or information summaries, reports, or other information related to this study may be revealed or released to any third party without prior notification and authorization of the sponsor.

ANNEX

A. Group assignment: Table –A – 106 mimotope

Mouse Order	Punch	Cage #	Mouse Order	Punch	Cage #	Mouse Order	Punch	Cage #
1	"0"	300 µg, 1A	**37**	"L1"	500 µg, 2A	**73**	"R1"	500 µg, 3A
2	"0"	Control, 1A	**38**	"L1"	Control, 2A	**74**	"R1"	300 µg, 3A
3	"0"	100 µg, 1A	**39**	"L1"	300 µg, 2A	**75**	"R1"	100 µg, 3A
4	"0"	500 µg, 1A	**40**	"L1"	100 µg, 2A	**76**	"R1"	Control, 3A
5	"L1"	300 µg, 1A	**41**	"R1"	100 µg, 2A	**77**	"B1"	500 µg, 3A
6	"L1"	Control, 1A	**42**	"R1"	Control, 2A	**78**	"B1"	100 µg, 3A
7	"L1"	100 µg, 1A	**43**	"R1"	300 µg, 2A	**79**	"B1"	Control, 3A
8	"L1"	500 µg, 1A	**44**	"R1"	500 µg, 2A	**80**	"B1"	300 µg, 3A
9	"R1"	300 µg, 1A	**45**	"B1"	500 µg, 2A	**81**	"0"	Control, 3B
10	"R1"	500 µg, 1A	**46**	"B1"	100 µg, 2A	**82**	"0"	100 µg, 3B
11	"R1"	Control, 1A	**47**	"B1"	300 µg, 2A	**83**	"0"	300 µg, 3B
12	"R1"	100 µg, 1A	**48**	"B1"	Control, 2A	**84**	"0"	500 µg, 3B
13	"B1"	500 µg, 1A	**49**	"0"	300 µg, 2B	**85**	"L1"	Control l, 3B
14	"B1"	300 µg, 1A	**50**	"0"	500 µg, 2B	**86**	"L1"	100 µg, 3B
15	"B1"	100 µg, 1A	**51**	"0"	100 µg, 2B	**87**	"L1"	500 µg, 3B
16	"B1"	Control, 1A	**52**	"0"	Control, 2B	**88**	"L1"	300 µg, 3B
17	"0"	300 µg, 1B	**53**	"L1"	100 µg, 2B	**89**	"R1"	500 µg, 3B
18	"0"	500 µg, 1B	**54**	"L1"	500 µg, 2B	**90**	"R1"	Control, 3B
19	"0"	Control, 1B	**55**	"L1"	Control, 2B	**91**	"R1"	100 µg, 3B
20	"0"	100 µg, 1B	**56**	"L1"	300 µg, 2B	**92**	"R1"	300 µg, 3B
21	"L1"	Control, 1B	**57**	"R1"	Control, 2B	**93**	"B1"	100 µg, 3B
22	"L1"	100 µg, 1B	**58**	"R1"	100 µg, 2B	**94**	"B1"	300 µg, 3B
23	"L1"	500 µg, 1B	**59**	"R1"	300 µg, 2B	**95**	"B1"	500 µg, 3B
24	"L1"	300 µg, 1B	**60**	"R1"	500 µg, 2B	**96**	"B1"	Control, 3B
25	"R1"	500 µg, 1B	**61**	"B1"	500 µg, 2B			
26	"R1"	100 µg, 1B	**62**	"B1"	Control, 2B			
27	"R1"	Control, 1B	**63**	"B1"	300 µg, 2B			
28	"R1"	300 µg, 1B	**64**	"B1"	100 µg, 2B			
29	"B1"	100 µg, 1B	**65**	"0"	300 µg, 3A			
30	"B1"	Control l, 1B	**66**	"0"	Control, 3A			
31	"B1"	500 µg, 1B	**67**	"0"	100 µg, 3A			
32	"B1"	300 µg, 1B	**68**	"0"	500 µg, 3A			
33	"0"	100 µg, 2A	**69**	"L1"	100 µg, 3A			
34	"0"	500 µg, 2A	**70**	"L1"	300 µg, 3A			
35	"0"	300 µg, 2A	**71**	"L1"	500 µg, 3A			
36	"0"	Control, 2A	**72**	"L1"	Control, 3A			

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B. Group assignment: Table –A- 107 mimotope

Mouse Order	Punch	Cage #	Mouse Order	Punch	Cage #
1	"0"	300 µg, 1A	**37**	"0"	300 µg, 2B
2	"0"	Control, 1A	**38**	"0"	500 µg, 2B
3	"0"	500 µg, 1A	**39**	"0"	Control, 2B
4	"L1"	300 µg, 1A	**40**	"L1"	500 µg, 2B
5	"L1"	Control, 1A	**41**	"L1"	Control, 2B
6	"L1"	500 µg, 1A	**42**	"L1"	300 µg, 2B
7	"R1"	300 µg, 1A	**43**	"R1"	Control, 2B
8	"R1"	500 µg, 1A	**44**	"R1"	300 µg, 2B
9	"R1"	Control, 1A	**45**	"R1"	500 µg, 2B
10	"B1"	500 µg, 1A	**46**	"B1"	500 µg, 2B
11	"B1"	300 µg, 1A	**47**	"B1"	Control, 2B
12	"B1"	Control, 1A	**48**	"B1"	300 µg, 2B
13	"0"	300 µg, 1B	**49**	"0"	300 µg, 3A
14	"0"	500 µg, 1B	**50**	"0"	Control, 3A
15	"0"	Control, 1B	**51**	"0"	500 µg, 3A
16	"L1"	Control, 1B	**52**	"L1"	300 µg, 3A
17	"L1"	500 µg, 1B	**53**	"L1"	500 µg, 3A
18	"L1"	300 µg, 1B	**54**	"L1"	Control, 3A
19	"R1"	500 µg, 1B	**55**	"R1"	500 µg, 3A
20	"R1"	Control, 1B	**56**	"R1"	300 µg, 3A
21	"R1"	300 µg, 1B	**57**	"R1"	Control, 3A
22	"B1"	Control l, 1B	**58**	"B1"	500 µg, 3A
23	"B1"	500 µg, 1B	**59**	"B1"	Control, 3A
24	"B1"	300 µg, 1B	**60**	"B1"	300 µg, 3A
25	"0"	500 µg, 2A	**61**	"0"	Control, 3B
26	"0"	300 µg, 2A	**62**	"0"	300 µg, 3B
27	"0"	Control, 2A	**63**	"0"	500 µg, 3B
28	"L1"	500 µg, 2A	**64**	"L1"	Control l, 3B
29	"L1"	Control, 2A	**65**	"L1"	500 µg, 3B
30	"L1"	300 µg, 2A	**66**	"L1"	300 µg, 3B
31	"R1"	Control, 2A	**67**	"R1"	500 µg, 3B
32	"R1"	300 µg, 2A	**68**	"R1"	Control, 3B
33	"R1"	500 µg, 2A	**69**	"R1"	300 µg, 3B
34	"B1"	500 µg, 2A	**70**	"B1"	300 µg, 3B
35	"B1"	300 µg, 2A	**71**	"B1"	500 µg, 3B
36	"B1"	Control, 2A	**72**	"B1"	Control, 3B

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C. Animal Sacrifice order: Table-B- 106 mimotope

Cage #	Sacrifices order
100-2A	1
100-3B	1
100-3A	2
100-1B	2
100-1A	3
100-2B	3
300-1A	1
300-1B	1
300-3A	2
300-3B	2
300-2A	3
300-2B	3

Cage #	Sacrifices order
500-3A	1
500-3B	1
500-1A	2
500-1B	2
500-2A	3
500-2B	3
Control-2A	1
Control-1B	1
Control-3A	2
Control-3B	2
Control-1A	3
Control-2B	3

D. Animal Sacrifice order: Table-B- 107 mimotope

Cage #	Sacrifices order
300-3A	1
300-3B	1
300-2A	2
300-1B	2
300-1A	3
300-2B	3

Cage #	Sacrifices order
500-1A	1
500-3B	1
500-2A	2
500-1B	2
500-3A	3
500-2B	3
control-1A	1
control-1B	1
control-2A	2
control-3B	2
control-3A	3
control-2B	3

E. Urinalysis assignment: Table-C- 106 mimotope

Cage-punch	Assignment		Cage-punch	Assignment		Cage-punch	Assignment
100-3A-O	8		100-2A-O	3		100-1A-O	5
100-3A-L1	1		100-2A-L1	2		100-1A-L1	2
100-3A-R1	4		100-2A-R1	4		100-1A-R1	3
100-3A-B1	2		100-2A-B1	6		100-1A-B1	6
100-1B-O	7		100-3B-O	5		100-2B-O	7
100-1B-L1	3		100-3B-L1	7		100-2B-L1	1
100-1B-R1	5		100-3B-R1	1		100-2B-R1	4
100-1B-B1	6		100-3B-B1	8		100-2B-B1	8
300-1A-O	4		300-2A-O	5		300-3A-O	5
300-1A-L1	3		300-2A-L1	8		300-3A-L1	6
300-1A-R1	5		300-2A-R1	6		300-3A-R1	2
300-1A-B1	6		300-2A-B1	1		300-3A-B1	3
300-1B-O	1		300-2B-O	4		300-3B-O	7
300-1B-L1	2		300-2B-L1	2		300-3B-L1	1
300-1B-R1	8		300-2B-R1	3		300-3B-R1	8
300-1B-B1	7		300-2B-B1	7		300-3B-B1	4
500-1A-O	4		500-2A-O	2		500-3A-O	4
500-1A-L1	1		500-2A-L1	7		500-3A-L1	7
500-1A-R1	3		500-2A-R1	5		500-3A-R1	3
500-1A-B1	5		500-2A-B1	4		500-3A-B1	5
500-1B-O	6		500-2B-O	1		500-3B-O	8
500-1B-L1	7		500-2B-L1	3		500-3B-L1	2
500-1B-R1	8		500-2B-R1	6		500-3B-R1	1
500-1B-B1	2		500-2B-B1	8		500-3B-B1	6
Control-1A-O	4		Control-2A-O	2		Control-3A-O	7
Control-1A-L1	7		Control-2A-L1	7		Control-3A-L1	6
Control-1A-R1	3		Control-2A-R1	8		Control-3A-R1	8
Control-1A-B1	5		Control-2A-B1	3		Control-3A-B1	1
Control-2B-O	8		Control-1B-O	5		Control-3B-O	5
Control-2B-L1	6		Control-1B-L1	6		Control-3B-L1	4
Control-2B-R1	1		Control-1B-R1	1		Control-3B-R1	3
Control-2B-B1	2		Control-1B-B1	4		Control-3B-B1	2

Note: *Gray areas represent the mice that are selected for urinalysis*

F. Urinalysis assignment: Table-C- 107 mimotope

Cage-punch	Assignment		Cage-punch	Assignment		Cage-punch	Assignment
300-1A-O	4		300-2A-O	5		300-3A-O	5
300-1A-L1	3		300-2A-L1	8		300-3A-L1	6
300-1A-R1	5		300-2A-R1	6		300-3A-R1	2
300-1A-B1	6		300-2A-B1	1		300-3A-B1	3
300-1B-O	1		300-2B-O	4		300-3B-O	7
300-1B-L1	2		300-2B-L1	2		300-3B-L1	1
300-1B-R1	8		300-2B-R1	3		300-3B-R1	8
300-1B-B1	7		300-2B-B1	7		300-3B-B1	4
500-1A-O	4		500-2A-O	2		500-3A-O	4
500-1A-L1	1		500-2A-L1	7		500-3A-L1	7
500-1A-R1	3		500-2A-R1	5		500-3A-R1	3
500-1A-B1	5		500-2A-B1	4		500-3A-B1	5
500-1B-O	6		500-2B-O	1		500-3B-O	8
500-1B-L1	7		500-2B-L1	3		500-3B-L1	2
500-1B-R1	8		500-2B-R1	6		500-3B-R1	1
500-1B-B1	2		500-2B-B1	8		500-3B-B1	6
Control-1A-O	4		Control-2A-O	2		Control-3A-O	7
Control-1A-L1	7		Control-2A-L1	7		Control-3A-L1	6
Control-1A-R1	3		Control-2A-R1	8		Control-3A-R1	8
Control-1A-B1	5		Control-2A-B1	3		Control-3A-B1	1
Control-2B-O	8		Control-1B-O	5		Control-3B-O	5
Control-2B-L1	6		Control-1B-L1	6		Control-3B-L1	4
Control-2B-R1	1		Control-1B-R1	1		Control-3B-R1	3
Control-2B-B1	2		Control-1B-B1	4		Control-3B-B1	2

Note: Gray areas represent the mice that are selected for urinalysis

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G. Standard Operating Procedure (SOP) Deviation Form

Breast Cancer Reseach

Standard Operating Procedure (SOP) Deviation Form

Employee Information			
Name:			
Employee ID:		Department:	
Job Title:		Phone:	
Manager:			
SOP Number/ Title/ Version			

SOP Deviation Details
All deviations to a SOP are to be reported immediately. Complete this form and give it to Dr. Thomas Kieber-Emmons in the Office of Breast Cancer Research in the Bio-Medical II building Room 306 at the University of Arkansas Medical Science (UAMS) or fax it to him at **501-526-5934**.
Who or What caused the deviation to the SOP?
Please describe the SOP deviation.
What effect did the deviation have on the study?
What do you do to rectify the deviation?
Who did you report the deviation to?
How did you report the Deviation?

Signature:		Date:	

H. Protocol Deviation Form

Breast Cancer Reseach

Protocol Deviation Form

Employee Information			
Employee Name:			
Employee ID:		Department:	
Job Title:		Phone:	
Manager:			
Protocol Title and Version			

Protocol Deviation Details
All deviations to the Protocol are to be reported immediately. Complete this form and give it to Dr. Thomas Kieber-Emmons in the Office of Breast Cancer Research in the Bio-Medical II building Room 306 at the University of Arkansas Medical Science (UAMS) or fax it to him at **501-526-5934**.
Who or What caused the deviation to the Protocol?
Please describe the protocol deviation.
What effect did the deviation have on the study?
What do you do to rectify the deviation?
Who did you report the deviation to?
How did you report the Deviation?

Signature:		Date:	

Animal examination record

Table of contents

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A. Weighing form (measurement in g)

Cage identification______________________________________

	Week										
Mice ID	1	2	3	4	5	6	7	8	9	10	11
0											
L1											
R1											
B1											
Initial & Date											

	Week										
Mice ID	12	13	14	15	16	17	18	19	20	21	
0											
L1											
R1											
B1											
Initial & Date											

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B. Immunization schedule Form

Cage identification____________________

Test article Name: _______________________

Test article concentration: ________________

	Immunization #1		Immunization #2		Immunization #3		Immunization #4		Immunization #5	
Mouse ID	Date	Initial	Date	Initial	Date	Initial	Date	Initial	Date	Initial
L1										
R1										
B1										

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C. Observation Form for site injection reaction

Group & Mouse identification________________________________

Observation of redness (**1**), swelling (**2**), heat (**3**), ulceration (**4**), hair loss at the injection site (**5**) or Normal aspect (**N**)

Write the number corresponding to the observation or N for absence of site injection reaction.

Week	Observation#1 / Date	Initial	Observation#2/ Date	Initial	Observation#3/ Date	Initial
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						

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D. Animal sacrifice schedule Form

Cage identification_______________________________

	Sacrifice			
Mouse ID	Date	Weight	Initial	Comment
O				
L1				
R1				
B1				

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E. Organ weighing form

Cage identification ____________________________

Date________________ Time________________

	Organ weighing (measurement in g)							
Mouse ID	Liver	Initial	Spleen	Initial	Kidney L/R	Initial	Heart	Initial
O								
L1								
R1								
B1								

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F. Gross Pathology Form

Animal identification_______ Prosector ______________ Date & Time___________

Organs	Examined Y/N	Normal Y/N	Comments
Spleen			
clitoral gland			
Ovaries			
Uterus			
Urinary Bladder			
Vagina			
Rectum			
Colon Gross lesions			
Cecum			
Ileum			
Jejunum			
Duodenum			
Mesenteric lymph nodes			
Pancreas			
Stomach			
Left and Right adrenal glands			
Left and Right Kidneys			
Liver			
Salivary glands, left and right, Parotid			
Submandibular lymph nodes, left and right			
Tongue			
Trachea			
Thyroids, left and right			
Esophagus			
Thymus			
Lungs			
Heart			
Skin, ventral and dorsal			
Skeletal muscle, quadriceps, left			
Femur, left			
Eyes, left and Right			
Brain Gross lesions			

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Vaccine
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Preclinical studies of carbohydrate mimetic peptide vaccines for breast cancer and melanoma

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Abstract

Limited immune responses to tumor-associated carbohydrate antigens (TACA) are due in part to their being self-antigens. Immunization with xenoantigens of TACA provides an approach to break tolerance and augment responses to TACA. Carbohydrate mimetic peptides (CMPs) as xenoantigens can induce serum antibodies that target shared carbohydrate residues on differing carbohydrate structures. In preclinical studies, we observe that CMP immunization in mice induce immune responses that are effective in inhibiting the in vitro and in vivo growth of breast cancer and melanoma tumor cells expressing self-target antigens. CMPs of TACA can be further defined that induce IgM antibodies with broadened responses to both breast and melanoma cells. Consequently, CMPs are effective at generating a multifaceted carbohydrate-reactive immune response that should be clinically evaluated for their ability to amplify carbohydrate immune responses against circulating or disseminated tumor cells.

Keywords: Gangliosides; Peptide mimotopes; Tumor vaccine; Breast cancer; Melanoma

1. Introduction

Cancer vaccines provide an avenue to generate sustained tumor specific immunity. Among the classes of tumor rejection antigens are tumor-associated carbohydrate antigens (TACA) for which antibodies are the primary mediators of tissue destruction [1–3]. Carbohydrate targeting tissue rejection is best typified by the natural antibody response directed against the α-Gal antigen, a major barrier in porcine-to-human xenotransplantation [4]. α-Gal tissue rejection supports a mechanistic rationale for targeting TACA as tumor-induced antibody responses in general resemble autoimmune responses [5]. The potential impact of TACA vaccines is demonstrated in clinical trials where patient survival significantly correlates with TACA-reactive IgM levels [6–9].

Some TACAs are glycosphingolipids (GSL) [10]. Antibodies that recognize GSLs such as the gangliosides GD2, GM2 and the Lewis Y (LeY) antigen are demonstrated to mediate complement-dependent cytotoxicity (CDC) and have been suggested to be more cytotoxic to tumor cells than antibodies that recognize protein antigens or TACAs-linked to protein antigens [11] that kill tumor cells by antibody dependent cellular cytotoxicity (ADCC). Antibodies to GD2 [12], and GM2 [13] are also able to mediate apoptosis of tumor cells. GSL-induced responses could augment naturally occurring TACA-reactive IgM antibodies that trigger apoptosis of tumor cells [14]. Consequently, optimizing sustained immunity against TACAs might have a beneficial effect on the course of malignant disease.

A variety of approaches are being taken to generate responses to TACA. Carbohydrate mimetic peptides (CMPs) of TACA are one approach. The characterization of CMPs

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is at present limited to preclinical studies. CMPs have been described for GD2 [15–17], GD3 [18], sialylated Lewis a/x [19], and LeY [15,20,21]. Importantly, in preclinical prophylactic and therapeutic vaccination studies, CMPs were efficacious in eliciting immune responses that reduced tumor burden and inhibited metastatic outgrowth [21–23]. CMPs can induce antibodies targeting common carbohydrate moieties on tumor cells of different origin and can be designed to induce antibodies with broad or specific TACA reactivities. Thus, CMPs represent a new and very promising tool to increase the efficiency of the immune response to glycan antigens. Here, we summarize some of the salient features associated with developing these novel immunogens for their translation into the clinic as breast cancer and melanoma vaccines.

2. Materials and methods

2.1. Mice and immunization

Animal studies have been reviewed and approved by the Institutional Care and Use Committee of the University of Arkansas for Medical Sciences. Peptides were synthesized as multiple antigen peptides (MAPs, Research Genetics, Huntsville, AL) made by FMoc synthesis on poly-L-lysine groups resulting in the presentation of eight peptide clusters [21]. Six to eight week-old BALB/c or C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbour, ME). Separate groups of mice were immunized three times at 2-week intervals via subcutaneous injection with 100 μg of the respective MAP and 20 μg QS-21 suspended in 100 μl of sterile PBS. Control animal groups received only 20 μg QS-21.

2.2. Flow cytometry

Staining, acquisition and analysis was performed as described earlier [24]. Briefly, cells were incubated with dilutions of mouse sera for 30 min and then stained with FITC-conjugated anti-mouse antibody for another 30 min on ice. Mean fluorescence intensity and percentage of positive cells were calculated from duplicates for each serum dilution.

2.3. ELISA assays

ELISA was performed as described before [21]. Briefly, plates were coated with gangliosides (3 μg/ml) dissolved in ethanol. After blocking of the surface, sera were added and incubated for 1 h at 37 °C. Mean absorbance was calculated from duplicates for each serum dilution and the dilution yielding half-maximal binding was determined.

2.4. Complement-dependent cytotoxicity

CDC was measured using GD2-expressing murine cell line B16F10 (ATCC) as previously described [21]. Briefly, 10 μl of cell suspension (4×10^4 cells/ml) were added to triplicate wells of a microtiter plate to which was added sera, followed by incubation on ice. Rabbit complement (Sigma) 1:4 was added and allowed to incubate for 4 h at 37 °C. Medium was discarded and cells were fixed using methanol. The number of viable cells was determined by Giemsa staining and the percentage of cytotoxicity was calculated. The assay was performed in duplicate with medium, sera, and complement controls.

2.5. Necropsy, histopathology, and in situ apoptosis detection

Fourteen days after the last vaccination, animals were euthanized via overdose of CO_2. A complete necropsy was performed and organs were placed immediately into 10% neutral buffered formalin (NBF). Tissues were processed and embedded in paraffin and sectioned at 6 μm. Sections were stained with hematoxylin and eosin for routine histologic evaluation.

2.6. Statistical analysis

Data were expressed as arithmetic means ± S.D. Means were compared using Student's t test. Differences between groups were considered significant if the p was <0.05. Saturation binding curves (of the type: conc. × maximal level/(K + conc.) + background) were fitted to the experimental data with the help of the non-linear regression unit of the STATISTICA for Windows (STATSOFT, Tulsa, OK). The parameter K was used as a measure of the concentration yielding half-maximal binding.

3. Results

3.1. CMPs target shared carbohydrate residues on differing carbohydrate structures

CMP sequences were first described with a high prevalence of tryptophan and tyrosine residues that were identified to be associated with differing carbohydrate moieties [20,25–28]. The sequence similarities that define these peptides suggest that antibodies to homologous peptides might cross-react with similar subunits expressed on what are otherwise dissimilar carbohydrate structures. Based upon these motifs we constructed CMPs that antigenically mimic the LeY antigen expressed on human breast cancer cell lines and also a lipid associated, structurally related difucoganglioside (6B ganglioside) [29], expressed on murine Meth-A fibrosarcoma cells and on human tumor cells [21]. In particular CMP 106 (H-GGIYWRYDIYWRYDIYWRYD)8-MAP and CMP 107 (H-GGIYYRYDIYYRYDIYYRYD)8-MAP induce antibodies reactive with LeY expressed on breast cancer cell lines and with Meth-A fibrosarcoma cells expressing

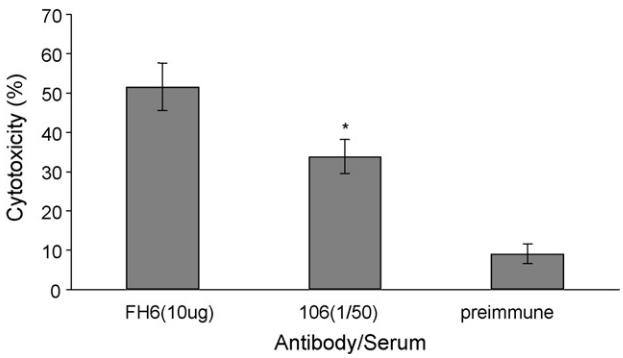

Fig. 1. CDC of B16F10 cells by mAb FH6 and 106 induced serum. Mice were immunized with CMP 106 and serum was collected after third immunization. *$p < 0.02$ compared with preimmune serum using Student's *t* test.

the 6B antigen [21]. The basis for this cross-reactivity is a shared Fucα1-3GlcNAcβ1-3Galβ1 epitope as suggested by conformational analysis of these peptides interacting with the antibody-combining site of the BR55-2 monoclonal [30]. CMP 106 is effective in vivo in prophylactic vaccination followed by challenge with Meth-A tumor cells [21].

The 6B antigen is also expressed on the murine B16F10 melanoma cell line. Both FH6 and serum antibodies induced by CMP 106 mediate CDC of this cell line (Fig. 1). We have also adopted a B16F10 lung metastasis model in which FH6 reactive B16F10 cells were used in challenge experiments after vaccination with CMP 106 (Fig. 2). As observed, the number of lung colonies were less in the CMP-immunized mice compared with naïve tumor bearing animals ($p < 0.05$). Collectively, these results, combined with our previous studies, indicate that CMP 106 induces serum antibodies that target TACA expressed on murine models of fibrosarcoma [21], breast cancer [22], and melanoma.

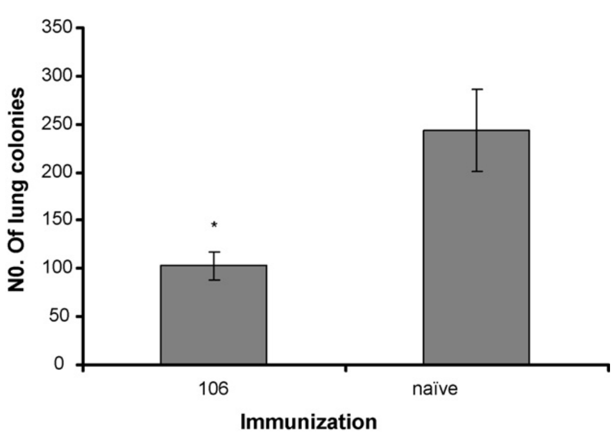

Fig. 2. Prophylactic vaccination inhibits lung colonization of B16F10 cells. C57BL/6 mice (groups of four) were immunized 3X with peptide 106 and QS-21. Mice then were challenged with 5×10^5 per mouse of B16 cells by tail vein injection. Fourteen days later the mice were sacrificed, lungs were removed, and tumor colonies were counted. Two separate groups of experiments were conducted. Error bars are estimated based on eight individual mice. *Fewer number of lung colonies as compared with naïve mice at $p < 0.05$ using Student's *t* test.

3.2. Defining CMPs that induce serum antibodies to multiple carbohydrate antigens

Gangliosides are typically considered expressed with neuroectodermal tumors (melanoma and neuroblastoma) but can also be expressed on breast cancer cells. The composition of cell surface gangliosides is largely dependent on the relative activities of Golgi resident glycosyltransferases. Transfer steps leading to synthesis of the gangliosides GM3, GD3, GM2 and GD2 from LacCer are functionally coupled in the Golgi membranes [31]. Individual carbohydrate-based vaccines are in clinical trials targeting these important TACAs. Another approach would be to synthesize several different tumor-associated carbohydrate antigens into a single molecule. Alternatively, antibodies can be induced by CMPs with broad specificity towards these gangliosides.

The monoclonal antibody ME36.1 whose crystal structure is known [32], displays reactivity with GD2 and GD3 [33]. Using the program LIGPLOT [34], primary hydrogen bonding partners are illustrated in the recognition of the GalNAc moiety by ME36.1 residues Thr H33, Asn H59 and Asp H50, the Gal moiety by ME36.1 residues Thr H33, His 35 and Ser 100H, Neu5Aca2-3 residue by Ser 100H and Neu5Aca2-8 residue by Tyr L93 (Fig. 3). This primary interaction scheme suggests that ME36.1 could react with GD2 and GD3, and perhaps with GM2, GM3, GD1b and GD1a. Such broad specificity for these important tumor-associated TACA in fact has been argued for using ME36.1 in the clinic [33] and emphasizes the importance of inducing multiple specificity towards tumor antigens, i.e., binding of an antibody to two or more TACA. Consequently, defining CMPs reactive with ME36.1 might in turn induce antibodies with broader ganglioside reactivity.

Screening a random peptide phage display library with the anti-GD2 ganglioside monoclonal antibody ME36.1 defined another WRY containing peptide with the sequence GVVWRYTAPVHLGDG and referred to as P10 [15]. ELISA analyses of serum antibody induced by the P10 CMP, also synthesized as an 8-mer MAP, suggest that immunization might enhance preexisting ganglioside reactive IgM serum antibodies (Fig. 4A, empty bars). Comparing the ratios of the dilutions yielding half-maximal binding for naïve and immune sera, we observe about a three-fold increase in GD1a and GD1b reactivity (GD1b is related to the GD3/GD2 synthetic pathway while GD1a is related to GM3/GM2/GM1 pathway). This is equivalent to increasing the titer to the same fold degree. We found a correlation between the IgM titer thus measured before and after immunization implying that the reactivity induced probably depended on the one already existing or that the CMP stimulated preexisting carbohydrate-reactive B cells (Fig. 4B, crosses). These data suggest that B cells capable of responding to GD1a and GD1b exist in an innate pool that can be polyclonally activated and/or in a B cell pool that can be specifically amplified by CMP. On the other hand, peptide immunization

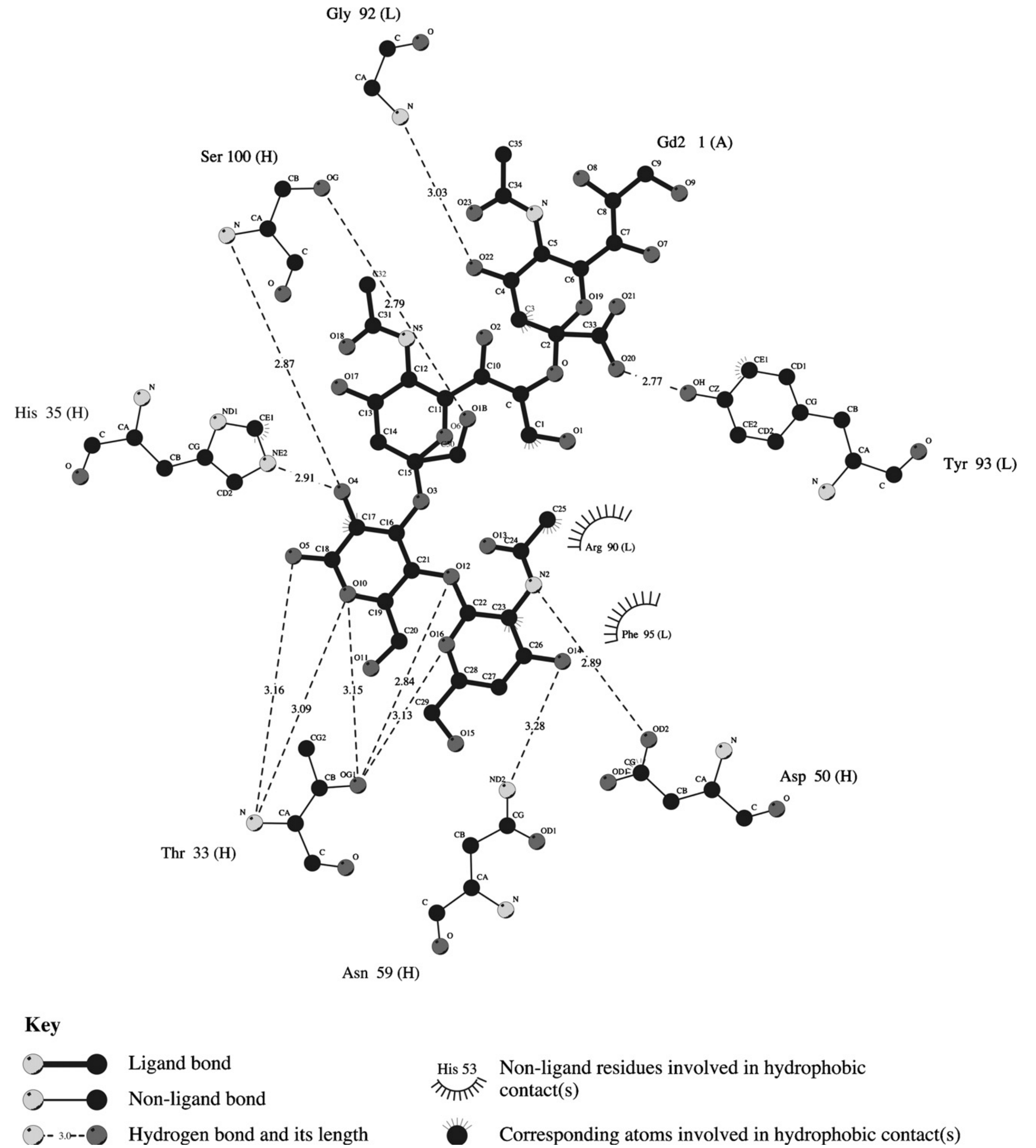

Fig. 3. LIGPLOT of GD2 recognition by ME36.1. Docking was performed using FlexX followed by energy optimization (Tripos). The network of hydrogen bonds and hydrophobic forces involved in stabilizing GD2 binding to ME36.1 are shown by two-dimensional plot using LIGPLOT [34]. This structure was of lower energy with more extensive hydrogen bonding than that originally reported [32].

did not boost significantly the preexisting IgM anti-GD2 response although P10 was selected by a GD2 binding antibody.

The ELISA results suggest that P10 might induce serum IgM antibodies reactive with multiple gangliosides. To further test this hypothesis we examined the binding of serum antibodies induced by P10 to the human breast cancer cell line MDA-231 and MCF7 (Fig. 5) as MDA-231 cells are purported to differentially express a variety of gangliosides relative to MCF7 cells [35]. As observed by flow cytometry, serum IgM antibodies bound considerably stronger to MDA-231 cells compared to MCF7 cells (Fig. 5).

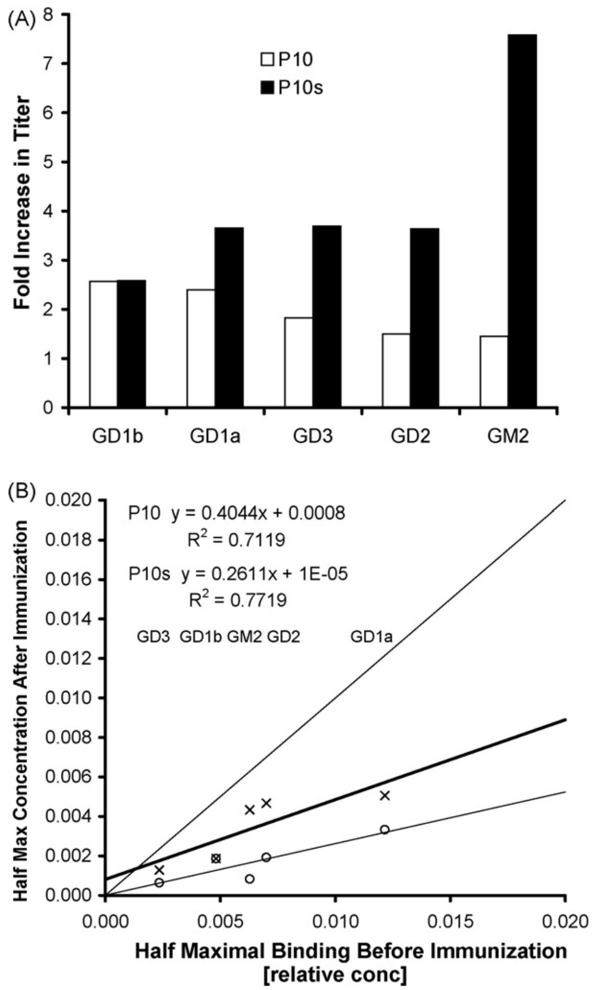

Fig. 4. Binding of serum IgM antibodies to gangliosides from mice immunized with P10 or P10s as well as from naïve mice. (A) Comparison of dilutions yielding half-maximal binding to different gangliosides. (B) Correlation between reactivity of naïve and immune sera to different gangliosides. The diagonal line marks the positions of the points indicating that immunization would not change the titer. The lines below this diagonal indicate increase in titer due to immunization. The slope of the line may be used as a measure of the sensitivity of the immune titers to the preimmune titers. (×) Data points for P10 and (○) data points for P10s.

3.3. Structural basis for CMP mimicry

Understanding the origin and structural basis for antibody responses arising in response to TACA through molecular mimicry is of critical importance to understanding anti-tumor response and the mechanisms by which tolerance operates. To understand how CMP P10 might functionally mimic GD1a, GD1b and GD2 we used conformational and energy analysis to define potential binding modes of this peptide in the crystallographically defined ME36.1 binding pocket (Table 1) [36].

Molecular modeling of the P10 CMP in the ME36.1 binding site indicates that this CMP only has two hydrogen bonds in common with the GD2 antigen in binding to ME36.1 (Table 1). This would suggest that serum raised to this peptide might be skewed to cross-react with gangliosides emphasizing Neu5Aca2-3 Gal residues shared with GD1a as Thr33 and Ser 100H are observed to interact with these two carbohydrate moieties. Terminal saccharides are typically viewed as dominant epitopes. Consequently, reactivity with the terminal Neu5Ac-α2-3Gal-β1 epitope might be a basis for reactivity with GD1a in that it has two potential epitopes. The modeling also suggests the GalNAc1-4Gal epitope might also be mimicked by P10 as Thr33 and SerH100 are contacting the Gal and GalNAc moieties on GD2 as well. However, it is not clear if orientation matters, which would explain the GD1b reactivity as there are potentially two residues that interact with Gal and only one interacting with GalNAc. We are exploring this possibility.

To test the hypothesis that CMPs can be redesigned to increase the level of mimicry, we developed a peptide with the sequence WRYTAPVHLGD (referred to as P10 short or P10s) with an increased number of hydrogen bonds relative to its parent peptide GVVWRYTAPVHLGDG. The redesigned P10s shares five hydrogen bonds with GD2 in binding to ME36.1 (Table 1). The increased number of hydrogen bonds would suggest that the antibody population induced by P10s might be redirected towards GD2 reactivity. We observe that P10s immunization changes the preexisting anti-ganglioside reactivity of serum from that of immunization with P10 (Fig. 4A), augmenting the response to GD2, GD3 and GM2. Serum IgM induced by P10s is observed to selectively bind to GD2 expressing melanoma WM793 cells (Fig. 6).

3.4. CMPs induce anti-tumor responses with absence of normal tissue damage

It is generally recognized that the pathology observed from tumor-reactive antibodies can mirror autoimmune-mediated tissue damage and antibody-inducing antigens can serve as rejection antigens if they are recognized as foreign [5]. We observe that CMPs 106, 107, P10, or P10s induce antibodies that mediate tumor growth inhibition without tissue damage to normal murine tissues that express the model antigens including brain and kidney (Fig. 7). The relative expression density of carbohydrate antigen on the surface of a tumor cell is generally suggested as a mechanism that diminishes normal tissue damage. In keeping with these general concepts our histopathology studies suggest that CMPs do not induce antibodies that mediate normal tissue destruction.

We did observe mild liver inflammation with immunization with CMPs P10 and P10s (Fig. 8). Minimal to mild lymphocytic portal hepatitis and mild lobular lymphoplasmacytic hepatitis with rare intralesional apoptotic hepatocytes were present in all groups. The mild degree of inflammation is consistent with the lack of clinical signs and normal weight gain observed in these mice. Further tests including evaluation of serum chemistries for levels of hepatic enzymes to detect sub-clinical hepatocellular damage are planned. Importantly, no inflammatory or demyelinating lesions were

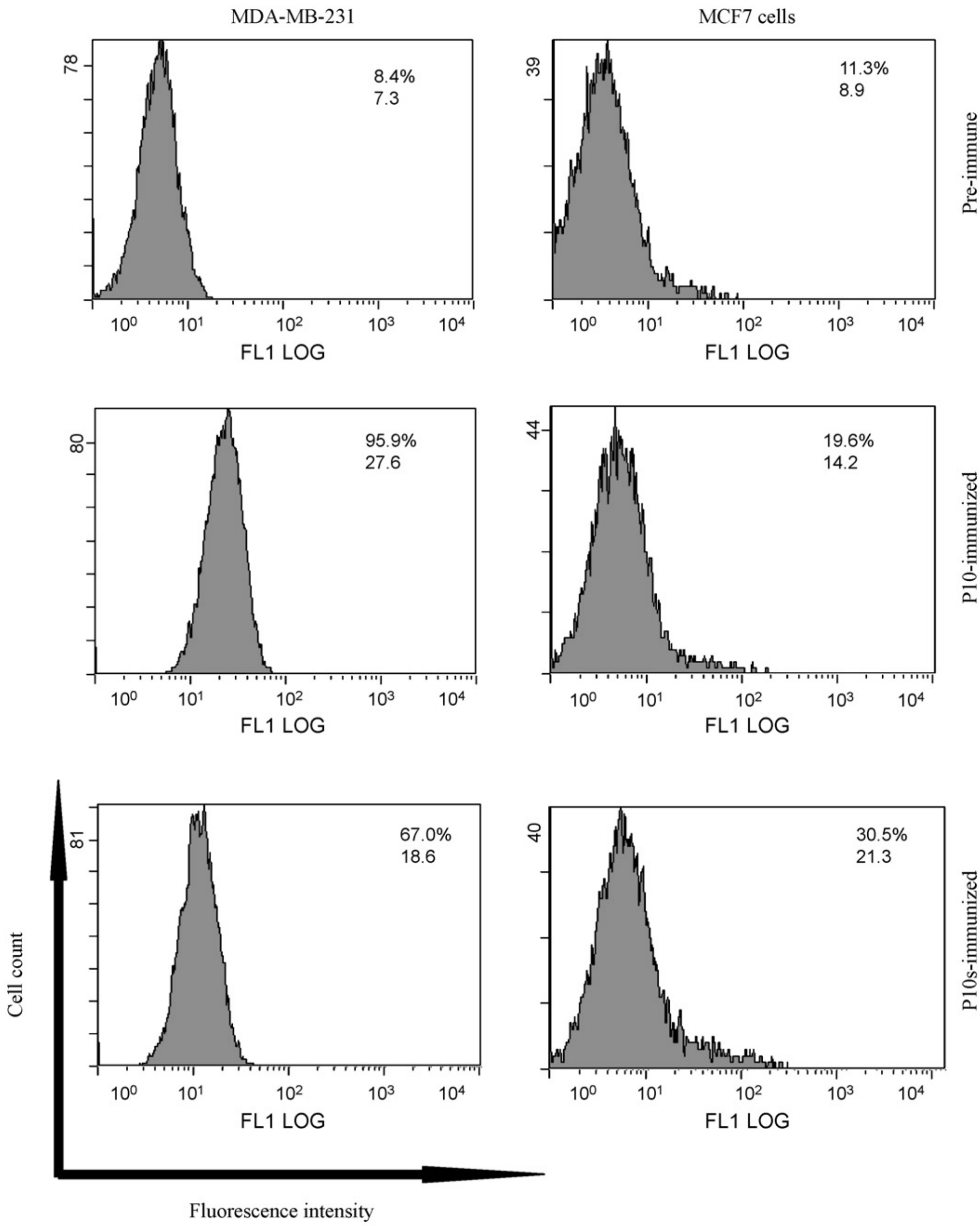

Fig. 5. Binding of serum IgM antibodies to MCF-7 (right column) and to MDA-MB-231 (left column) cells. Groups of mice (C57BL/6) were prebled and then immunized with P10 and P10s peptides. Serum was collected 7 days after the fourth immunization and pooled for each group. Cells were harvested using enzyme-free buffer and binding of serum IgM antibodies to cells was detected by flow cytometry. Percentage of positive cells (upper number) and MFI of whole population (lower number) for each histogram is shown. The reactivity of 1:200 dilution of serum is shown. X and y axes show relative fluorescence intensity and cell number, respectively.

Table 1
Modification of flanking residues enhance GD2 mimicry for ME36.1 binding

Ligands[a]	ΔG (free binding energy) (kJ/mol)	Residues on ME36.1						
GD2	−10.06	H/Thr33	H/His35	H/Asp50	H/Asn52	H/Asn59	L/Tyr93	H/Ser100
GVVWRYTAPVHLGDG	−17.60	H/Thr33						H/Ser100
WRYTAPVHLGDG	−50.00	H/Thr33		H/Asp50		H/Asn59	L/Tyr93	H/Ser100

[a] The mimotope residues forming hydrogen bonds with ME36.1/GD2 contact residues are underlined.

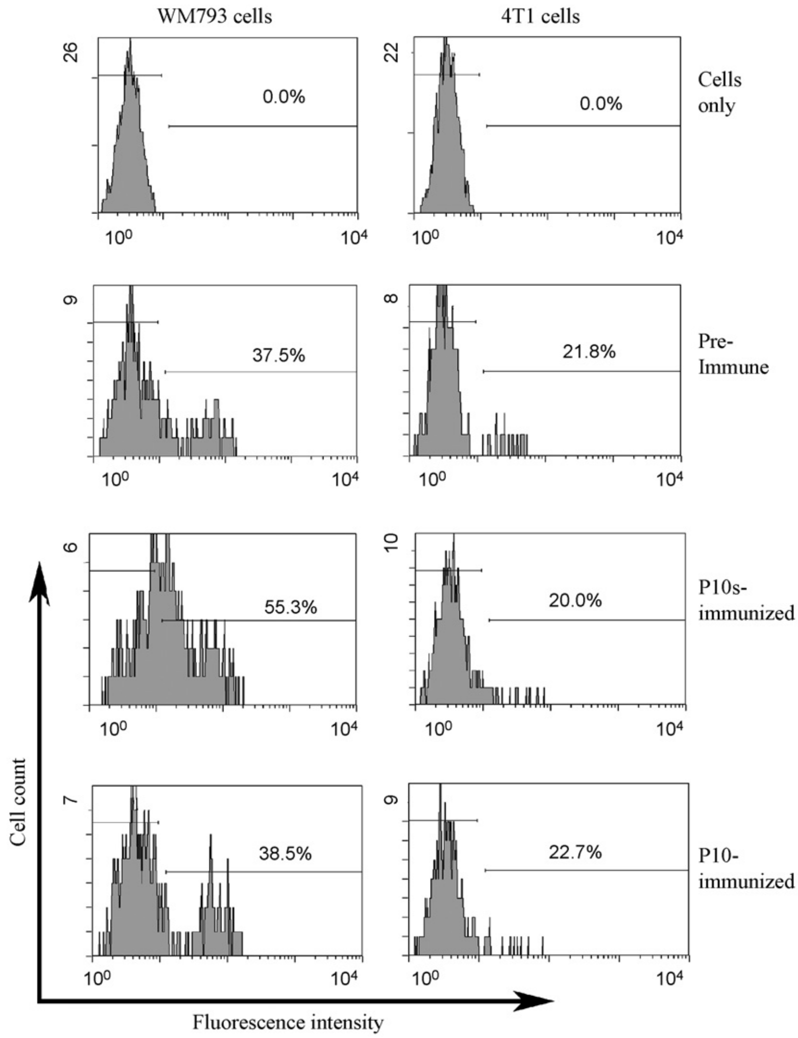

Fig. 6. Comparison of serum binding to GD2 expressing cells. Mice (BALB/c) were prebled and then immunized with P10 short (P10s) peptide. Serum was collected 7 days after the third immunization and pooled for each group. Cells were harvested using enzyme-free buffer and binding of serum IgM antibodies (1:50 dilution) to cells was detected by flow cytometry. Percentage of positive cells for each histogram is shown. X and y axes show relative fluorescence intensity and cell number, respectively.

noted in nervous tissue, which generally has the highest tissue expression of gangliosides (data not shown). Normal mice appear to be resistant to neural injury during anti-GD1a antibody exposure, demonstrating the central role of membrane antigen density in modulating both immune tolerance to GD1a and axonal susceptibility to anti-GD1a antibody mediated injury [37]. In keeping with these general concepts our histopathology studies suggest that CMPs do not induce antibodies that mediate normal tissue destruction targeting gangliosides due to their low density of expression.

4. Discussion

We have made several striking discoveries in developing CMPs. (1) CMP-induced serum antibodies can be functionally related to the natural antibodies that represent an innate immune surveillance system; (2) the identification of pre-existing antibodies to CMP might also indicate preceding encounter of cross-reactive antigens and a possibility to elicit a swift recall response to TACA that suppresses the metastatic process; (3) immunization with CMPs is effective in eradicating cancer cells and impacting on tumor metastases without inducing significant immunopathology.

Circulating carbohydrate-reactive IgM antibodies are proposed as a mechanism of immune surveillance of the innate immune system [14]. Such antibodies might be designated as natural antibodies because of their ubiquitous occurrence with no basis for assignment of immunogenic induction. The fact that survival rates of cancer patients are correlated with low avidity and low titer carbohydrate-reactive antibodies [6–9] argues that having more robust IgG responses may not be of absolute necessity.

The same or similar TACAs are expressed on both breast cancer and melanoma cells. CMPs can induce IgM antibodies that target TACAs on breast cancer and melanoma

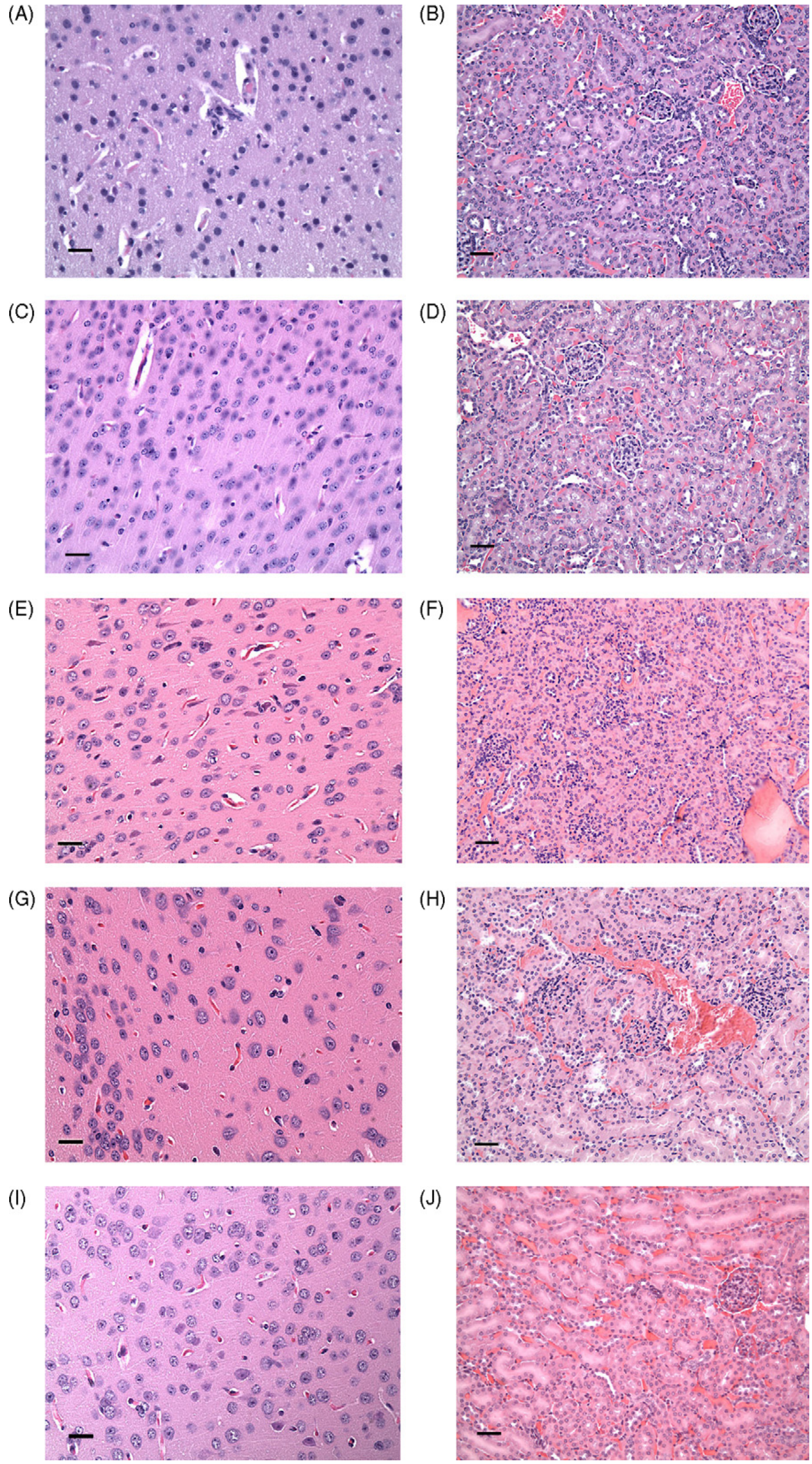

Fig. 7. Immunization with CMPs does not induce immunopathology. CMP 106 (A and B); CMP 107 (C and D); P10 (E and F); P10s (G and H); naïve control (I and J). Left column: cerebrum, 400× magnification, bar equals 40 μm. Right column: kidney, 200× magnification, bar equals 80 μm. H&E stain.

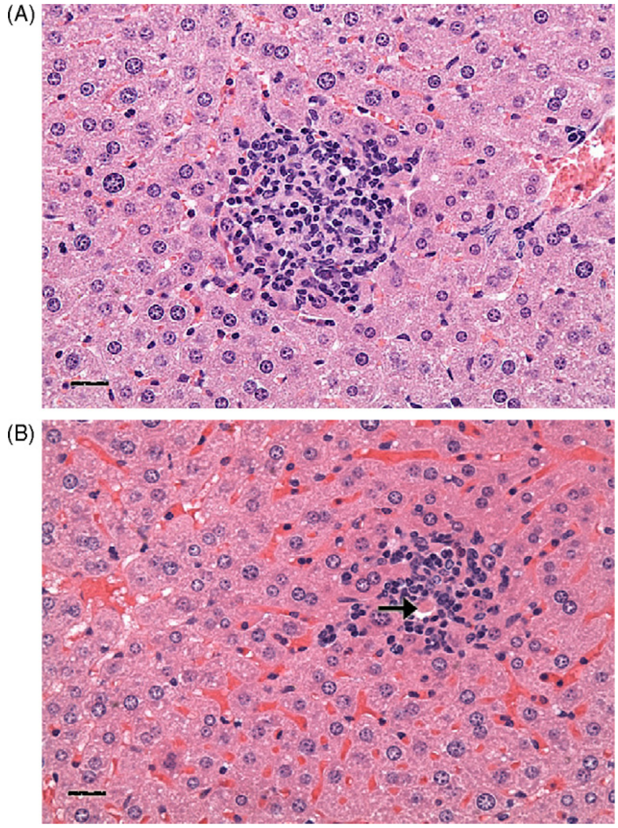

Fig. 8. Mild liver inflammation in mice vaccinated with P10s (A) and P10 (B). Tissues were fixed in 10% neutral buffered formalin, processed and embedded in paraffin, sectioned at 6 μm, stained with hematoxylin and eosin, and examined under a light microscope. An apoptotic hepatocyte (arrow) is demonstrated, indicating hepatocellular damage. H&E, 400× magnification. Bar equals 40 μm.

cells. Several of these TACAs are gangliosides that are shared with the nervous system such as GM1, GM2, GM3, GD2, and GD3. Natural anti-tumor immunity triggered by tumor-related gangliosides could be at the origin of immune-mediated peripheral neuropathies when the epitope is shared by tumor and the peripheral nervous system. However, we observe that non-tumor bearing mice have preexisting IgM antibodies reactive gangliosides that are enhanced particularly upon P10s-CMP immunization suggesting that the ganglioside reactive IgM must have their origin among B2 cells (probably marginal zone B cells). In parallel, preexisting ganglioside reactive antibodies have been noted in normal healthy individuals [9]. Ravindranath et al. [9] have argued that circulating tumor-gangliosides might be perceived as danger signals by the host's immune system as evidenced by the endogenous antiganglioside immune response to gangliosides. They went on to suggest that the endogenous IgM against gangliosides may facilitate elimination of these danger signals to restore immune competence of the host. Our results suggest that CMPs can augment these endogenous responses. While we did not observe any evidence of demyelination upon immunization with any of the CMPs, IgM antibodies to gangliosides are attributed as a contributing factor to multifocal motor neuropathy in some cancer patients and therefore must be monitored in clinical trials.

It is anticipated that CMPs can sustain TACA reactive responses in breast cancer and melanoma patients, facilitating long-term immunosurveillance through recall of carbohydrate immune responses that should contribute to patient survival. Preclinical studies support the hypothesis that vaccine-induced responses against TACA might have their greatest impact in the adjuvant setting as such responses inhibit tumor outgrowth in metastatic models [22,38]. The induction of long-lived responses capable of eradicating cancer metastases suggests that vaccines could be effective against tumor recurrence. However, unlike pathogen infections that provide "danger signals" to the immune system, cancer cells are rather a source of tolerance signals and therefore constant boosting of the immune surveillance and possibly suppression of Treg function may be warranted.

Acknowledgments

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Carbohydrate mimetic peptides induce tumor-associated carbohydrate-reactive antibodies in the absence of pathological autoimmunity[1]

Running title: Carbohydrate mimetic peptides do not induce autoimmunity

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Abstract:

Purpose: Cancer vaccines are argued to facilitate tissue damage in a manner akin to the induction of autoimmunity. Carbohydrate targeting tissue damage is best typified by the natural antibody response directed against the alpha-Gal epitope, a major barrier in porcine-to-human xenotransplantation. To demonstrate that autoimmunity is not an inevitable consequence of amplification of carbohydrate reactive antibodies, the immune pathology of BALB/c mice immunized with two carbohydrate mimetic peptides of tumor associated carbohydrate antigens (TACAs) potent enough to induce anti-tumor response and reactive with ubiquitously expressed self carbohydrates on murine tissues was analyzed.

Experimental Design: Tissues from unimmunized mice were labeled with *Griffonia simplicifolia* lectin 1 (GS-I) and antibody to murine IgG to demonstrate the presence of natural, circulating antibodies against terminal galactose. Western blots of membranes from murine mammary 4T1 cells, syngeneic with BALB/c mice, were compared using GS-I lectin, immunized serum antibodies, and naive serum antibodies. Tissues from immunized mice were analyzed histologically after 4 immunizations and after 1 year of immunization using hematoxylin and eosin stain, TUNEL stain for apoptosis, and Luxol-fast blue staining for myelination. ELISA against ssDNA, dsDNA, and histones was performed on sera from these mice.

Results: The pattern of expression of terminal galactose moieties is restricted and is closely paralleled by the immunoglobulin deposition pattern in unimmunized mice. There was no evidence of pathological autoimmunity in any immunized mice. Titers of clinically relevant antinuclear antibodies were not significantly elevated.

Conclusions: These results demonstrate that vaccination with carbohydrate mimetic peptides can enhance antibodies to TACAs without inducing immunopathology.

Introduction

Development of effective therapeutic vaccines to prevent tumor recurrence is of great clinical interest (1). While the design of many cancer vaccines focus solely on the induction of cellular immune responses (2, 3), increasing evidence from both animal and human studies indicates that antibodies are effective in tumor cell destruction (4-7). It is generally recognized that the pathology observed from tumor-reactive antibodies can mirror autoimmune-mediated tissue damage and antibody-inducing antigens can serve as rejection antigens if the Tumor Associated Antigens (TAAs) are recognized as foreign (8). Tumor-targeting antibodies are however, frequently detected in cancer patients without signs of autoimmunity (9-11). Because low levels of antibodies able to react with most normal tissues can be identified in normal donors, concern is raised that as the level of these antibodies increases, autoimmunity may develop (9).

Tumor associated carbohydrate antigens (TACAs) have been described as potential targets in cancer vaccine development (10). Carbohydrates belong to a class of TAAs for which antibodies are the primary mediators of tissue destruction. The best-known example of carbohydrate targeting tissue damage is the natural antibody response directed against the alpha-Gal carbohydrate antigen, a major barrier in porcine-to-human xenotransplantation (14-18) Anti-Gal antibodies are known to be deleterious as organ rejection is mediated in large part by alpha-Gal-reactive antibodies directed at endothelium that mediate complement activation as a mechanism of tissue damage. TACA-directed antibodies could mediate tissue damage similar to that caused by alpha-gal-reactive antibodies, which could lead to tumor regression or participate in immune surveillance. Carbohydrate-reactive IgM antibodies in the naive repertoire have been described that bind predominantly to carbohydrates on post-transcriptionally-modified antigens, that induce apoptosis and, most importantly, detect not only malignant cells but also premalignant stages (11, 12). While boosting the frequency of such naturally occurring circulating carbohydrate-reactive antibodies or enhancing the potency of TACA directed vaccines with such activity increases concern regarding the potential induction of autoimmune pathologies in immunized individuals, investigations on TACA expression continue to suggest that these antigens are weakly expressed or inaccessible to the immune system in non-neoplastic tissues.

As a means to augment immune responses to TACAs we have developed carbohydrate mimetic peptides (CMPs). In separate sets of experiments, we demonstrated that the immune response induced by two CMPs in particular and referred to as CMPs 106 and 107, are effective in tumor regression in mouse models of cancer (13-15). These CMPs function as surrogate immunogens for several TACA that include the neolactoseries antigen Lewis Y (LeY)(13) expressed exclusively in humans, a difucosylated ganglioside antigen called 6B (13) that is expressed in murine and human tumors (16) and terminal galactose determinants like alpha-gal (14, 15) which are expressed on tissues of many animals (17-20). The CMP 107 in particular functions as an alpha-Gal and terminal galactose mimic in that it binds to the *Griffonia simplifica* B4 (GS-1-B4) protein (14) and induces proapoptotic antibodies paralleling the proapoptotic activity of GS-1. Griffonia proteins recognize a broad array of carbohydrates that contain terminal-galactose (like alpha-Gal), in addition to LeY and Lewis B (LeB) (21, 22) and Tn antigen on human tumor cells (31-33).

In the present study we demonstrate that induction of TACA-cross-reactive B cells and T cells in mice upon immunization with CMPs 106 and 107 that mimic a broad spectrum of carbohydrate antigens including ubiquitously expressed terminal Galactose to levels sufficient to mediate therapeutic anti-tumor immunity *in vivo,* can occur without the development of adverse immunopathology to normal tissues. Because of the perception of the abundant and rather wide distribution of glycoconjugates with terminal-galactose, it is expected that binding by GS-I, and antibodies induced by CMP 107, should identify tissues containing glycoconjugates with terminal-galactose that are potentially immunologically accessible. Consequently, this is an excellent model system to evaluate the adverse immune response to a vaccination regimen that is potent enough to induce tumor therapy.

Methods

CMP synthesis and Immunization.

Animal studies have been reviewed and approved by the Institutional Care and Use Committee of the University of Arkansas for Medical Sciences. CMP 106 with the sequence GGIYWRYDIYWRYDIYWRYD and CMP 107 with the sequence GGIYYRYDIYYRYDIYYRYD were synthesized as multiple antigen peptides (MAPs) (Bio-Synthesis inc., Lewisville, TX), made by FMoc synthesis on poly-L-Lysine groups resulting in the presentation of eight peptide clusters (13). BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were randomly assigned to 4 groups (N=2). Two groups were immunized three times at two-week intervals via subcutaneous injection with either 100µg of Map 106 or Map 107 with 20µg QS-21 admixed with 20µg of keyhole limpet hemocyanin (KLH) in 100µl of sterile PBS as adjuvant. One control group received only 20µg QS-21 and KLH, and another control group left unvaccinated (naïve). To determine the effects of long-term, repeated exposure, a separate group of 4 mice was similarly immunized with 100µg of MAP 106 six times during a one-year period (long-term treatment group).

Western blotting.

Proteins (40 µg) were separated on gradient 4-12% SDS-PAGE (Invitrogen, Carlsbad, California) and transferred to polyvinylidene fluoride membranes (Invitrogen, Carlsbad, California). The membranes were blocked with 3% BSA containing 0.01% Tween 20 overnight in Phosphate Buffer Saline (PBS; pH 7.6) at 4°C and then incubated with serum (1:1000 dilution) or 2µg/ml of biotinylated GS-I lectin in PBS containing 1% BSA and 0.01% Tween 20 for 2h at room temperature. After the membranes were washed, they were incubated for 1 h at room temperature with peroxidase-conjugated streptavidin (0.25 ug/ml) or anti-mouse IgM antibodies (1:2000 dilution) in PBS containing 0.01% Tween 20. For visualization, an enhanced chemiluminescence based detection system (ECL, Amersham Pharmacia Biotech) was used and the membranes were exposed to X-ray film (Eastman KODAK Co; Rochester, NY).

Lectin histochemistry.

Sections were deparaffinized, hydrated through xylenes and graded alcohol series, and washed in DPBS. Antigen retrieval with 0.1 M citrate buffer (pH 6.0) for 40 min at 100ºC was performed. Endogenous peroxidase activity was blocked by immersion in 0.3 % (w:v) hydrogen peroxide in absolute methanol for 10 min followed by DPBS wash. Nonspecific binding was blocked by incubating with DPBS containing 2% BSA at RT for 35 min. Sections were then incubated with 2.5 µg/ml GS1-biotinylated for 1h at RT and washed in DPBS containing 0.1% Tween 20. Sections were then incubated with streptavidin horseradish peroxidase (Dako cytomation) for 30 min at RT, followed by incubation with DAB solution for 2 min at RT, then rinsed in distilled water. Slides were counterstained with hematoxylin for 30s, cleared and mounted. The biotinylated GS1 was omitted for negative control.

Immunohistochemistry for antibody deposition

Sections were deparaffinized, hydrated through xylenes and graded alcohol series, and washed in DPBS. Antigen retrieval with 0.1 % Trypsin (Mediatech Inc, Herndon, VA 20171) for 5 min at RT was performed. Endogenous peroxidase activity was blocked by immersion in 0.3 % (w:v) hydrogen peroxide in absolute methanol (Dako cytomation, Carpinteria, CA93013) for 10 min followed by DPBS wash. Nonspecific binding was blocked by incubating with DPBS containing

2% BSA at RT for 35 min. Sections were then incubated with a biotinylated mixture of anti-mouse IgG and anti-mouse IgM (Dako cytomation, Carpinteria, CA93013) for 1h at RT and washed in DPBS containing 0.1% Tween 20, followed by incubation with streptavidin horseradish peroxidase (1/5000, Pierce, Rockford, IL 61105) for 30 min at RT. Sections were then incubated with DAB solution (Zymed Laboratories Inc, South San Francisco, CA 94080) for 2 min at RT, washed in distilled water. Slides were counterstained with hematoxylin for 30s, cleared, and mounted.

Necropsy, histopathology, and in-situ apoptosis detection.

Fourteen days after the last injection, animals were euthanized via overdose of CO_2. Cardiac puncture was performed immediately post-mortem to obtain blood. A complete necropsy was performed and organs were placed immediately into 10% neutral buffered formalin (NBF). Organ weights were obtained for liver, heart, left and right kidneys, spleen, and lung. The brain was fixed in situ in 10%NBF. Tissues were processed and embedded in paraffin and sectioned at 6µm. Sections were stained with hematoxylin and eosin for routine histologic evaluation. Sections of brain and spinal cord were stained using the Luxol-fast blue technique to identify myelin.

In situ apoptosis detection was performed using the TUNEL technique using Apoptag peroxidase *In situ* Apoptosis Detection Kit S7100 (Chemicon International). Briefly, sections were deparaffinized, rehydrated, and treated with 20µg/ml proteinase-K for 15min at room temperature. Sections were washed with 2 changes of distilled H_2O for 2 min each. Endogenous peroxidases were blocked with 3%H_2O_2 in PBS for 5 min and washed with three changes of PBS. Equilibration buffer containing digoxigenin-conjugated nucleotides was placed directly on the sections for 10s. Sections were incubated with TdT enzyme in a humidified chamber at room temperature for 1 hour. Sections were then incubated for 10m in stop-wash buffer at room temperature, rinsed in 3 changes of PBS for 1min each, and incubated with anti-digoxigenin conjugate at room temperature for 30 min. Sections were washed in PBS, counterstained with 0.5% (w/v) methyl green, and evaluated under a light microscope. Incubation with active TdTs was omitted for negative controls. Sections treated prior to staining with DNAse served as positive controls.

ELISA.

Serum samples were prepared using heart blood from mice in the long-term treatment group and age-matched controls. Reactivity of serum against single stranded DNA (ssDNA), double-stranded DNA (dsDNA) and histone was determined. Briefly, plates were coated with ssDNA, dsDNA (Calbiochem, San Diego, CA) and histone (sigma), prepared from calf thymus, in Reacti-Bind™ DNA coating solution (Pierce, Rockford, IL) according to manufaturere instruction. Wells then were blocked using 200 µl/well of 1X PBS/FCS 10%. Serial dilutions of sera in 1X PBS/FCS 10 % were applied in 100µl volumes to wells and incubated overnight at 4ºC. Plates were washed 7 times with 1X PBS/Tween 0.5% and blotted dry on paper towel. 100µl of detecting antibody (Polyclonal Alkaline Phosphatase Conjugated Goat-anti-mouse IgG, IgA+ IgM (H+L), Zymed Laboratories, South San Franciso, CA) diluted 1:2000 in 1X PBS/FCS 5%. was added to each well and plates were incubated for 1 hr at room temperature. Plates were washed again 7 times with 1X PBS/Tween 0.5% and blotted dry on paper towel. Alkaline Phosphatase Substrate (Sigma, St. Louis, MO) was added to wells at 100µl/well. Plates were incubated at room temperature in the dark, and read at a wave length of 405 nm on an ELISA Plate Reader.

Statistical Analyses.

Student's t test was used to compare means. Differences were considered significant if P was < 0.05. All assays were repeated at least three times.

Results

Tissue distribution of GS-I binding is restricted

The GS-I isotypes GS-I-B4 and GS-I-A4 bind to group B and group A antigens, respectively, and exhibit strong binding to broadly expressed Gal1-2, Gal1-3 and Gal1-4 glycans (17).

Carbohydrate residues reactive with GS-I were previously shown to be present on the surface of highly-malignant murine tumors but absent or expressed in much lower amounts on the surface of low-malignant cells isolated from the same parent tumors (34-36). To further validate the expression pattern of GS-I on tumors, we implanted murine 4T1 cells into mammary fat pads of a group of mice in a separate experiment, and, at day 35 post-transplant, mice were euthanized. And sections of liver, lung and primary tumor mass were prepared and stained with GS-I (Fig. 1) This animal model closely resembles human breast cancer and is a rigorous model of advanced spontaneous metastatic disease, which metastasizes efficiently to lung, liver, bone and brain after implanting into mammary fat pads (37-40). Lung metastases were detected as early as day 14 after transplantation in all mice tested, whilst liver metastases were detected around day 28-35 after transplant, paralleling observations of others (23).. We observed enrichment in GS-I staining of tumor cells in sections of lung and liver compared to the primary tumor. Staining of normal tissues other than hematopoietic cells at 2.5ug/ml GS-I concentration was much less intense than the staining demonstrated in primary tumor sections and metastatic tumors at the same concentration (Fig. 2). These results suggest that tumor cells on both primary 4T1 tumors and their metastases are enriched for GS-I binding sites compared with normal tissues. Our findings are consistent with previous observations that GS-I binds to endothelial cells and neurons of rodents and other animals (26-28, 16, 41). GS-1 reactive alpha-gal epitopes on endothelial cells are presumably readily accessible to the immune system.

Similar cell-expressed epitopes are reactive with GS-I and antibodies

Although CMPs 106 and 107 induce antibodies with complement-dependent-cytotoxicity (CDC) activity (24), CMP 107 also induces proapoptotic antibodies for 4T1 and the human breast cancer cell line MCF7 (14). CMP 107, and not CMP 106, competes with antigen for GS-I binding, indicating that CMP 107 binds to GS-I (14). The proapoptotic activity of the CMP 107-induced antibodies parallel the apoptotic activity observed upon GS-I binding of these cells, suggesting that apoptotic induction is via CMP 107- antibody recognition of GS-I ligands (14).

SDS-PAGE profiles of 4T1 cells with GS-I revealed several bands of varying molecular weight, some of which were also identified, albeit weakly, by natural IgM antibodies in the naive (non-immunized) repertoire (Fig. 3). Western blot analyses with CMP 107-induced serum antibodies displayed a pattern that paralleled the pattern of preimmune antibodies with intensified bands (Fig. 3), suggesting that immunization with CMP 107 may amplify a preexisting naïve repertoire.Our results suggest that immunization with CMP 107 may amplify low-titer terminal-Gal reactive antibodies that are effectively mimicking GS-1 reactivity for 4T1 cells.

To further define the tissue reactive properties of naive antibodies, staining was performed to detect if immunoglobulin deposition occurred on normal tissue of unvaccinated mouse control tissues. As with GS-1 staining, deposition of immunoglobulin was detected on endothelial cells in the blood vessels of the kidney, liver, brain, and heart (Fig. 4). Antibody deposition was not present outside the vasculature in these organs. In contrast, immunoglobulin deposits were detected on tumor cells and surrounding stroma (Fig. 4). These data suggest that preimmune antibodies display an epitope and tissue distribution pattern similar to GS-1 and that they might be amplified upon CMP immunization.

Immunization with CMPs does not lead to immunopathology

Because immunization with CMPs 106 and 107 induce anti-tumor immune responses, H&E stained sections of organs including the liver, kidney, heart, lungs, intestines, stomach, lymph nodes, spleen, brain, spinal cord, and eyes were examined to determine whether significant immunopathology was observed after immunization with either CMP 106 or 107. No significant cellular infiltrates were identified in any organ, including the brain and spinal cord, from any animal in the short-term treatment groups, and there was no evidence of necrosis or extensive apoptosis in these sections. Because alpha-Gal antigen is highly expressed in neural tissues (Fig. 1), brain and spinal cord are tissues likely to be most affected by immunopathology caused by formation of antibodies against this antigen. In animals immunized at various intervals for one year, changes observed, including lymphocytic and plasmacytic renal infiltrates, were within normal limits for mice of this age.

To rule out subtle changes in myelination we examined serial brain and spinal cord sections stained with Luxol fast blue for myelin. There was no difference in myelination between immunized and control animals (data not shown). TUNEL stain was performed on serial sections of all organs. Apoptosis was rare in all sections, and there was no detectable difference between CMP-immunized and control animals (data not shown).

Previous studies demonstrated that a significant lymphoid infiltrate was associated with regressing tumors upon CMP 106 immunization (15). CMP 106 is hypothesized to functionally mimic GlcNAc expressed on glycopeptides associated with MHC Class I, which is recognized by T cells (15). In contrast, we noted that cellular infiltrates were not found in normal tissues of animals vaccinated with CMP 106 further indicating that infiltrating T cells induced by CMP 106 are tumor-specific. Collectively these data demonstrate that although the titers of tumor cell-reactive antibodies induced in the CMP 106 and 107-immunized animals would be sufficient to cause tumor growth inhibition (21, 23), histological analyses indicate no evidence of immunopathology in normal tissues.

A feature of many auto-immune disorders is the formation of antinuclear antibodies. We examined possible subclinical deleterious consequences of immunization with the CMPs by testing for several pathognomonic autoreactivities in immunized mice. Antibodies against dsDNA, ssDNA, and histone were assayed in serum from all treatment groups. We did not observe significant reactivity with dsDNA or histone, however, a statistically significant reactivity of low titer (1:400) was observed against ssDNA with both anti-106 and anti-107 sera (Fig. 5). ssDNA is a major reactivity among the natural antibodies (25) and levels are believed to increase with age (44,45). Its clinical utility depends on defining appropriate cut off values to differentiate between normal levels and a pathological increase (26). In the clinic anti-ssDNA antibodies are measured in arbitrary units that are proportional to OD values determined in ELISA rather than by end-point titer. By this criterion the increase found after immunization with CMPs is borderline and comparable to the normal fluctuations of this parameter with age. In a separate set of experiments we confirmed this point and observed that immunization causes an increase in the reactivity of these antibodies as assessed by OD values in 5-month old mice, comparable to the levels found in 18-month old non-immunized mice (Fig. 6). We did not detect emergence of anti-DS or anti-histone serum antibodies even with serum tested at one year after immunization with continuous boosting (data not shown).

Discussion

The major challenge faced by cancer vaccines is that many potential tumor antigens also are found on normal cells. Therefore, it is perceived that immunization against cancer must overcome self to induce a state of tissue damage specifically targeting tumor cells without significant immunopathology to normal tissues (27, 28). Immune surveillance mechanisms are responsible for the removal of transformed cells, and antibodies play an important role in these immune processes. Carbohydrates have established themselves as the most clinically relevant antigens of those tested and subsequently developed for vaccines against infectious diseases, which might be translated to cancer vaccines. The potential impact of TACA-directed vaccines is demonstrated in clinical trials where patient survival significantly correlates with carbohydrate-reactive IgM levels (29). Such results suggest that TACA-targeting vaccines might have a beneficial effect on the course of malignant disease and TACA-induced responses could augment naturally occurring carbohydrate-reactive IgM antibodies that trigger the apoptosis of tumor cells (11, 12). A unique advantage in targeting TACAs is that multiple proteins and lipids on cancer cells can be modified with the same carbohydrate structure. Thus, targeting TACAs has the potential to broaden the spectrum of antigens recognized by the immune response, thereby lowering the risk of developing resistant tumors due to the loss of a given protein antigen (30).

CMPs of tumor antigen epitopes potentially represent a novel vaccine approach to induce a tumor antigen-specific humoral and cellular response and a strategy for inducing more robust immune responses to TACAs (51-55). Protein surrogates of TACAs are T-cell–dependent antigens and therefore immunization with these surrogates is predicted to facilitate cellular responses. CMPs have been described for GD2 (56-58), GD3 (31), sialylated Lewis a/x (32), and LeY (13, 33). Although the characterization of CMPs is at present limited to preclinical studies, clinical characterizations of anti-idiotypic antibodies that mimic the GD3 ganglioside antigen (34) and GD2 (35) have been described. In addition, unlike carbohydrate antigens and carbohydrate-conjugate vaccines, we have shown that CMPs prime B- and T-cells for subsequent memory of carbohydrate antigens, facilitating long-term surveillance through recall of carbohydrate immune responses (36).

As the CMPs 106 and 107 are functional mimics of a broad spectrum of TACA, it might be argued that they would induce antibodies that would lead to tissue damage. To test this hypothesis we examined the tissue distribution pattern of terminal Gal reactive with GS-1 and anti-CMP antibodies. We observed that GS-1 ligands are generally restricted to neurons, endothelial cells, and hematopoietic cells within the bone marrow. The increased level of GS-I binding on tumor cells demonstrates that GS-I reactive antigens are upregulated on tumor cells compared to normal cells. We observed that CMP 107 binds to GS-I and, in mice, immunization with CMP 107 seems to amplify a native repertoire of antibodies that bind to 4T1 cells. There was no evidence of inflammation, necrosis, or extensive apoptosis in any examined organ, and no significant differences were noted between control animals and vaccinated animals for either CMP. The absence of immunopathologies is particularly notable in neural tissues where the GS-1 reactive antigen is most strongly expressed. No significant changes in myelin or in the degree of myelination were noted.

Vaccination with these CMPs does not appear to lead to subclinical autoimmunity. We did not detect differences in the levels of the clinically relevant autoantibodies to dsDNA or histones. Our results indicate that a small increase in anti ssDNA is within normal variation, and is without clinical consequence in these mice. In pristane induced lupus ssDNA antibodies were found to be the first autoreactivity to appear (37), but appearance of these antibodies was followed within a couple of months by a diverse set of other pathological atuoreactivities. Thus, we hypothesize that the CMPs induced slightly increased levels of (natural) autoantibodies, but did not bring about overt autoimmunity. This is not surprising since the CMPs studied are designed to target B cell clones with specificities usually found in the preimmune repertoire. Increases in ANAs in aging C57BL/6 mice have been reported previously, and anti-ssDNA may be present in both humans and mice for long periods of time without clinical consequence (38). Indeed, we found in a separate experiment that the increase in anti-ssDNA induced by vaccination is within the range of natural anti-ssDNA antibodies in aging BALB/C mice (28, 39, 40). However, because anti SS DNA antibodies may indicate developing autoimmunity (41, 42), one should consider testing of anti-ssDNA in the patient population under vaccination therapy in clinical trials with CMPs.

Antibodies recognizing TAA can lead to tumor-targeted immunopathology and tumor destruction; in this sense, the humoral response to TAAs resembles that of other immune responses to self-antigens. Collectively our results demonstrate that repeated injections of CMPs 106 and 107 do not lead to immune-mediated injury in this preclinical study. These results are in concordance with reports from clinical trials using other types of cancer vaccines against these TACAs (43-48). It is estimated that in humans 1% of circulating immunoglobulin is represented by alpha-gal reactive antibodies (49). CMP 107, functioning as a GS-I epitope, could be expected to cross-react with circulating IgG reactive with the α-Gal epitope (14-18), but ELISA screening of healthy and breast cancer patients indicates the majority of these sera show no specific anti-106, or anti-107 reactivity (data not shown). This observation further suggests existence of multiple epitopes on structures presenting terminal α-Gal as well as that the CMPs define specific subsets among them while GS-1 has a broader reactivity including both human and mouse natural antibodies and CMP-like epitopes.

The underlying reasons for the absence of apparent immunopathology upon immunization with our xenoantigens is of interest. It is possible that the levels or patterns of expression of these TACA molecules on the surface of tumor cells differs significantly from that on normal cells. We demonstrated that IgG in naïve animals is deposited in the endothelium of several organs and in tumor cells, mirroring the binding of GS-1 and CMP immunization only amplifies moderately this reactivity. The recognition depends also on a threshold of avidity defined by the epitope's expression levels. Thus, fine specificity and quantitative thresholds are among a number of mechanisms that render immune tolerance resilient allowing for immunotherapy approaches that formally target self eptiopes. Antibodies induced by CMPs are thought to have low affinities for TACA. Thus, preferential targeting of tumor cells may be due in part to over-expression of the TACA on tumor cells, which compensates for the low affinity of the carbohydrate cross-reactive antibodies (50) and potential immunopathology due to destruction of normal tissue is minimized. Other features of the cell surface such as the three-dimensional arrangement of carbohydrate residues and characteristics of the protein such as size and valency may affect the expression patterns of TACAs on the cell surface and play critical roles in specific interactions on cell surfaces (51). It is known that TACAs tend to cluster on the surface of tumor cells (52), and these CMPs are designed to mimic that pattern.

In summary, this study supports the development of these CMPs for clinical testing. The ability of CMPs to induce antibodies reactive with multiple TACAs is relevant as heterogeneity of antigen expression in different cancers of the same type, as well as different cells of the same cancer, and heterogeneity of immune response in different patients makes it likely that maximal anticancer effect may not result from immunization against a single antigen. Consequently, immunizations with polyvalent vaccines containing several TACAs or immunization with CMPs that functionally emulate several TACAs are a viable strategy in vaccine development. Our results suggest that these vaccines will be safe for long-term treatment, and larger, preclinical safety studies are expected to verify these findings. Bringing these vaccines to the treatment armamentarium may significantly improve outcomes for patients affected with breast cancer and other types of tumors.

Footnotes:

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Figure Legends

Figure 1: **Histochemical staining of 4T-1 tumors:** Murine 4T-1 tumors were labeled with 2.5µg/ml GS-1 . Metastatic tumor cells in the liver (A) and lung (B) demonstrate increased GS-1 binding compared to cells within the primary tumor (C). 200x magnification, bars equal 40µm.

Figure 1

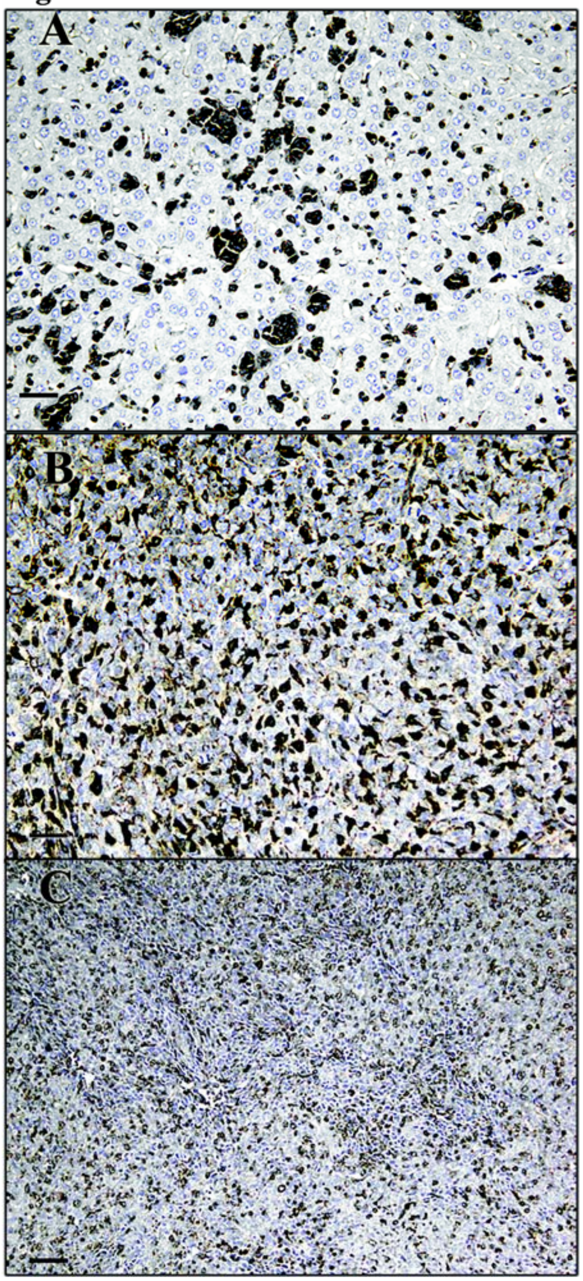

Figure 2:Tissues from a naïve control mouse were labeled with GS-1. A) Brain: Neurons (arrows) are labeled in a cytoplasmic and membranous fashion. B) Spinal cord: Neurons are labeled in a membranous pattern. C) Heart: Endothelial cells in the interstitium and in small arteries (arrows) are labeled. Myofibers (arrowheads) are not stained. D, E) Kidney, Liver: Endothelial cells (arrows) bind GS-1 weakly to moderately. Hepatocytes and renal tubular and glomerular epithelial cells do not bind GS-1. F) Bone marrow: Hematopoietic cells bind GS-1 strongly in a membranous and cytoplasmic pattern. A,B,C: 200x magnification, bar equals 40μm. D,E,F: 400x magnification, bar equals 20μm.

Figure 2

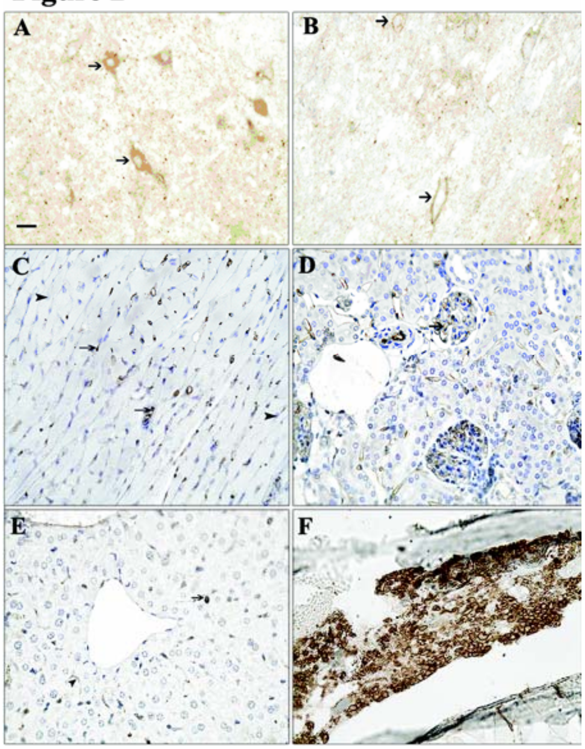

Figure 3. Serum and GS-1 binding to 4T1 cell lysate. Serum IgM antibodies and GS-1 binding to 4T1 cell lysate. Whole-cell lysate from 4T1 cell line was prepared. Mice (5/group) were pre-bled and immunized three times at two-week intervals with peptide 107. Sera were collected 7 days after the peptide boost and pooled for each group. Western blot analysis: Sera were diluted 1:1000 for the western. Anti mouse IgM used as secondary antibody. Binding with biotin-conjugated GS-I was followed by streptavidin-HRP.

Figure 3

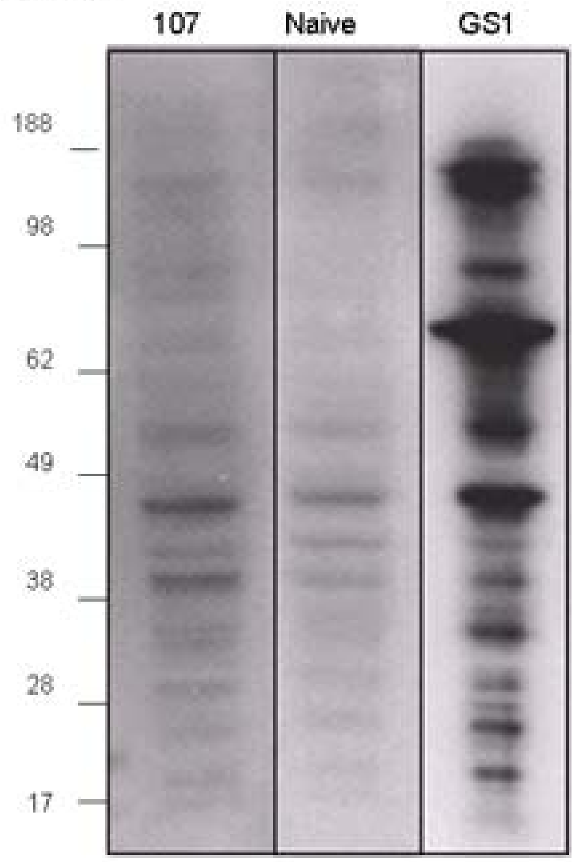

Figure 4. Histology of immunoglobulin on normal tissue. Normal tissues (A-C) and a murine 4T1 tumor (D) from naïve mice are immunostained with antibody against murine immunoglobulins. Endothelial cells in brain (A), kidney (B), liver (C), and primary tumor (D) are labeled with a distribution mirroring that of GS-1 binding. IgG is bound to tumor cells (arrows) and stroma (star) in the primary tumor (D). 400x magnification, bars equal 20µm.

Figure 4

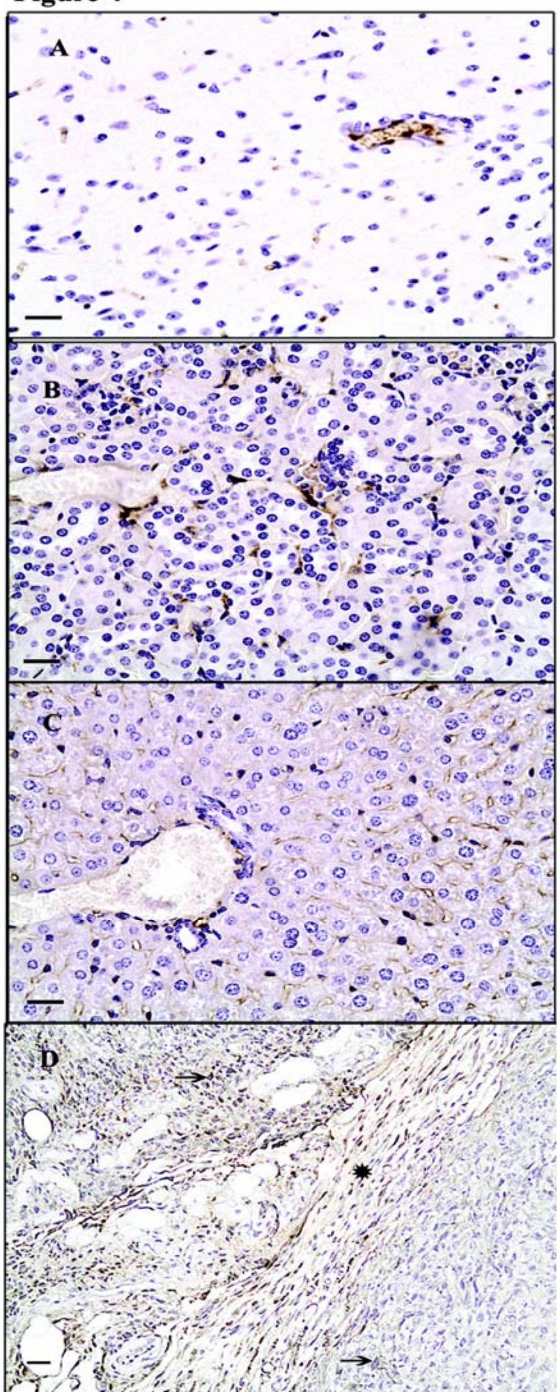

Figure 5. Mice serum binding to ss-DNA. OD values are significantly different up to 1/400 dilution compared to those of naive mice.
* p= 0.012 (106); p= 0.014 (107).
+ p= 0.001 (106); p= 0.005 (107).
# p= 0.015 (106); p= 0.007 (107).

Figure 5

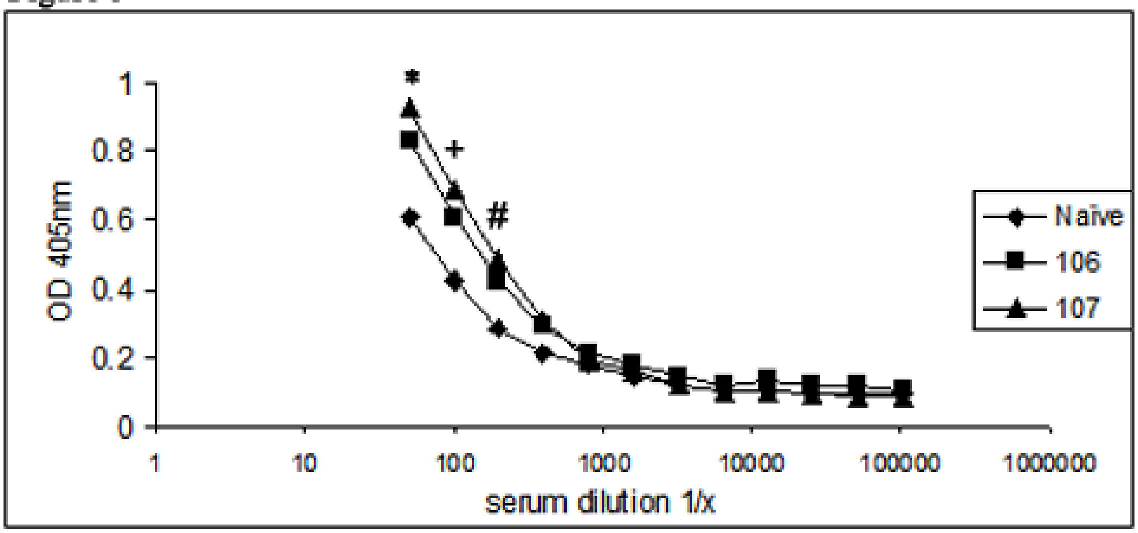

Figure 6. Mice serum binding to ss-DNA. OD values for pooled serum from mice immunized for 1 year with CMP106 or CMP 107 are similar to values observed in naive mice at 18 months of age.

Figure 6